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T_H1 cytokines induce senescence in AML

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ABSTRACT

Cancer testis antigen PRAME is over-expressed in a variety of malignant cells but is not or minimally expressed in normal non-germ line cells. Adoptive transfer of PRAME-specific T cells is thus under investigation in clinical trials as an innovative therapeutic option for acute myeloid leukemia (AML). However, their senescence-inducing activity has not been studied. This study therefore examines senescence induction in AML cells by PRAME-specific T_H1 cells. Analysis of cell cycle and marker expression demonstrate that the supernatants of antigen-stimulated PRAME-specific T_H1 cells induce senescence in AML cell lines Kasumi and Nomo-1 through combinative IFN-γ and TNF-α. Additionally IFN-γ and TNF-α secreted by TCR-activated $V\delta2^+$ or CMV-specific T cells can also drive these AML cell lines into terminal growth arrest. G1/0 arrest is also suggested in patient-derived AML by T_H1 cytokines or supernatants from Zoledronate-stimulated or aCD3/aCD28-stimulated PBMCs. Thus, we show for the first time that senescence is induced in AML cells by combined IFN-γ and TNF-α, and that these cytokines can be derived either from TCR-engineered $CD4^+$ T cells, or intriguingly from Virus-specific as well as innate $V\delta2^+$ T cells responding to their cognate antigens, namely T-cell responses targeting an antigen that is NOT expressed by the leukemic cells.

1. Introduction

Acute myeloid leukemia (AML) accounted for 1 Million deaths globally in 2017 [1]. Its prognosis remains poor; with a five-year overall survival rate below 30% [1]. Besides intensive chemotherapy as a mainstream therapeutic option, the adoptive transfer of T cells with tumor specificity has brought hope in recent years. T cells engineered to express T-cell receptors (TCRs) specific for tumor-associated antigens (TAAs) target Cancer-testis antigens (CTAs), normally expressed in germline tissues but aberrantly expressed in malignant cells. PRAME -one such CTA - is overexpressed on several solid cancers and hematologic malignancies [2,3] and two early phase clinical trials testing autologous T cells expressing PRAME/A* 02:01-specific TCRs for treatment of AML (NCT02743611, NCT03503968) are ongoing.

The overall effect of cellular senescence - the irreversible arrest of cell proliferation [4] - on treatment efficacy for AML is still controversial, since senescence is associated with the secretion of proinflammatory cytokines, chemokines, proteases, and growth factors, characterized as the senescence-associated secretory phenotype (SASP).

Chemotherapeutic agents can generate SASP in healthy tissue by inducing senescence with varying modes of action, creating a chronic inflammatory state with unwanted side effects supporting minimal residual disease and disease recurrence [5,6]. In contrast, senescence mediates desired effects as it inhibits the proliferation of malignant cells and stimulates immunosurveillance and therefore clearance of malignant cells [7,8]. Noteworthy key contributor to acute promyelocytic leukemia (APL) eradication by retinoic acid and arsenic trioxide, *the* curative drugs in the treatment of APL, is senescence induction in leukemic cells [9]. Therefore it is of critical importance to investigate senescence-inducing activity when evaluating a new therapeutic approach. However, it is still unknown whether TCR-engineered T cells can induce senescence in the antigen-expressing AML cells.

$CD4^+$ TCR-engineered T cells have recently been described to be able to induce senescence in tumor cells through T_H1 cytokine signals [10–12]. Although PRAME-specific $CD8^+$ CTLs for T cell transfer in hematological malignancies have been studied [13–16], PRAME-specific $CD4^+$ T cells have not been studied that intensively. The purpose of this study therefore was to generate PRAME-specific $CD4^+$ Th1 cells and to analyze whether PRAME-specific $CD4^+$ T cell-derived cytokines can induce senescence in PRAME-expressing AML cells. Moreover, to put cytokine-induced senescence in a broader context, we hypothesize that it may represent a general mechanism that protects

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inflamed tissue from the uncontrolled proliferation of stressed, dysregulated and transformed cells. Consequently, we sought to expose AML cells to supernatants derived from TCR-stimulated Virus-specific T cells as well as TCR-stimulated Vδ2$^+$ γδ T cells, innate T cells that convey integrity of the tissues, to elucidate whether cytokines derived from T-cell responses targeting an antigen that is NOT expressed by leukemic cells can also induce senescence in AML.

2. Methods

2.1. Bioethics

This study was performed in accordance with the ethical standards and recommendations of the institutional ethic committee of the Medical Faculty of the Eberhard-Karl-University and the University Hospital of Tübingen and was approved under references: 105/2017BO2. All subjects included gave written informed consent.

2.2. PBMC isolation and culture

Healthy volunteers' PBMCs were isolated via density gradient centrifugation (Biocoll Separating Solution; Biochrom, Germany). Written informed consent was obtained from each donor as described above.

2.3. AML cells from a patient

AML blasts were isolated before initial treatment from a 7-month-old male infant newly diagnosed with AML (M5) and used for analysis. Written informed consent was obtained from the donor`s parents as described above.

2.4. AML cell culture

Representative AML cell lines Nomo-1 (*MLL-AF9*-driven AML) and Kasumi (*AML1-ETO*-driven AML) were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), cultured in RPMI 1640 (Life Technologies) supplemented with 10% (Kasumi) or 20% (Nomo-1) heat-inactivated bovine calf serum, 1x GlutaMax (Gibco/Life Technologies), 1% sodium pyruvate (Gibco/Life Technologies), 1% ampicillin + streptomycin (Biochrom) at 37 °C, 5% CO_2.

2.5. Antigens for the generation of PRAME, CMV, and MOG-specific T cells

For in-vitro stimulation of PRAME-specific T cells, PepTivator PRAME (Miltenyi, order # 130–097–286) consisting mainly of 15-mer sequences with 11-amino-acids overlap, covering the complete sequence of PRAME antigen, was used. Myelin oligodendrocyte glycoprotein, a self-protein served as negative control (PepTivator ® MOG, Miltenyi, order # 130–096–770), superantigen staphylococcal enterotoxin B (SEB; Sigma-Aldrich # S4881) as a positive control for T-cell stimulation. PepTivator® CMV pp65 – premium grade (Miltenyi, order # MB130–093–435) was used for CD4$^+$ and CD8$^+$ T cell stimulation in PBMCs of CMV-positive donors.

2.6. Generation of supernatants from CMVpp65-stimulated PBMCs of CMV-positive tested donors

Donor PBMCs were isolated, resuspended in RPMI1640 (10% FCS, containing L-Glu, Pen/Strep) at 10^7 cells/ml, plated at 5×10^6/cm^2 in 24 wells with 2 ml/well. 20 µl of peptide-pool stock solution/ml was added (about 1 µg per peptide/ml) and cells cultivated for 7 days. Cells were restimulated day 4 and 7, harvested 4 h after last stimulation, centrifuged (450 G 5 min, 1000 G 15 min) and cell-free supernatants collected.

2.7. Generation of supernatants from zoledronate-stimulated PBMCs

Donor PBMCs were isolated, resuspended in RPMI1640 (10% FCS, containing L-Glu, Pen/Strep) at a concentration of 4×10^6/ml in the presence of IL-2 (30 U/ml), IL-15 (10 ng/ml) (both from Sigma-Aldrich) and 1.0 µM zoledronate (Zol, Selleckem, Munich, Germany) and cultivated for 7 days. On days 3 and 6, half of the supernatant volume was discarded and replaced with fresh medium containing cytokines. Because zoledronate specifically confers the conformational requirements for γδ TCR stimulation [17–19], zoledronate stimulation leads to the selective outgrowth of this T cell subtype. On day7, over 90% of the cells are CD3$^+$ with between 80% and 95% being Vδ2 γδ T cells.

2.8. Generation of PRAME-specific T cells

Dendritic cells (DCs) were generated as described previously [20]. Briefly, donors' PBMCs (2–5 $\times 10^6$ cells/ml) were incubated in AB-medium (37 °C, 2 h) to allow monocytes to adhere to the culture flask. After washing with warm PBS, IL-4 (40 ng/ml) and GM-CSF (100 ng/ml) were added to the cells (2–5 $\times 10^6$ cells/ml) to induce their differentiation (day 0). Day 6, IL-4 (40 ng/ml), GMCSF (100 ng/ml), IL-1β (10 ng/ml), IL-6 (100 ng/ml), TNF-α (10 ng/ml), and PGE22 (1 µg/ml) were added. Day 7, DCs were harvested and analyzed by flow cytometry for (CD80 and CD83) and T-cell marker CD3.

1×10^8 PBMCs were pre-sensitized to PRAME peptides (1 µg/ml, Miltenyi) in the presence of IL-2 (10 U/ml) and IL-7 (10 ng/ml) in AB-medium. Half medium was changed and cytokines added every other day. Day 7, 2.5×10^6 cells were harvested, restimulated with PRAME and frequency of IFN-γ-secreting T cells determined (IFN-γ-Secretion Assay-Detection Kit (PE) (Miltenyi). SEB (10 µg/ml, Sigma) served as a positive, MOG (1 µg/ml, Miltenyi) as a negative control. When frequency of IFN-γ-secreting, PRAME-specific T cells was > 0.1%, PRAME-specific T cells were enriched with a MACS separator (CliniMACS Cytokine Capture System (IFN-γ), Miltenyi). Non-IFN-γ-secreting cells (negative fraction) were irradiated (30 Gy) and used as feeder cells at a ratio of 100:1 (feeder: responder) for the expansion of PRAME-specific T cells over a period of 14 days (5×10^6 cells/ml, IL-2 (50 U/ml), IL-7 (10 ng/ml) IL-15 (10 ng/ml)). Culture splitting, medium change and cytokine supplementation were conducted every two days. Day 14, cells were harvested, 1.25×10^6 T cells used to determine cytokine secretion profile and memory phenotype and remaining cells were cryopreserved.

2.9. Collection of PRAME-specific T-cell supernatants

PRAME-specific T cells (5×10^6 cell/ml) were cultured in AB-medium with PRAME-peptide-pool pulsed DCs (5×10^5 cell/ml) for 5 days. T cells were restimulated 4 h with PRAME-peptide-pool pulsed DCs (5×10^5 cell/ml) then cells were centrifuged (450 G 5 min), supernatants re-centrifuged (1000 G 15 min) to collect cell-free supernatants, which were then shock frozen and stored at − 80 °C until use.

2.10. Reseeding experiments

2.5×10^5 tumor cells/ml were seeded in 24-well plates in medium or medium with IFN-γ (100 ng/ml) and TNF-α (20 ng/ml) and incubated at 37 °C, 5%CO_2. When medium of control (medium only) turned yellow, reseeding was performed. For that same number of "treated" as well untreated control cells were seeded into new wells, all in standard culture medium. This procedure was repeated up to 4 passages.

2.11. Flow cytometric analysis

Antibodies are listed in Supplementary Table 1. Intracellular staining was performed with the FIX&PERM® Cell Permeabilization Kit (ADG Bio Research, Austria), with cells cultured in the presence of 1 µg

Brefeldin A for 4 h before staining. Samples were measured using a BD FACS CantoII flow cytometer and analysed using Flow Jo Software.

2.12. Cell-cycle analysis

Nomo-1, Kasumi and patient AML M5 cells, were cultured in 48-well plates (5×10^5 cell/ml). Medium or medium with IFN-γ (100 ng/ml) or/ and TNF-α (20 ng/ml) or supernatants from unstimulated or PRAME-stimulated PRAME-specific $CD4^+$ T cells was added. 96 h later, cells were labelled with EdU using the Click-iT EdU Alexa-Fluor 647 Flow Cytometry Kit (ThermoFisher Scientific). 16 h later tumor cells were harvested and analyzed for cell-cycle phase with EdU/propidium iodide (PI) using a FxCycle™ PI/RNase Staining Solution (ThermoFisher Scientific).

2.13. Neutralizing antibodies

Anti-IFN-γ antibody (clone#25718, R&D; 50 ng/ml) and anti-TNF-α antibody (clone D1B4, Cell Signaling Technology, Danvers, MA, USA;10 ng/ml) were added to neutralize IFN-γ and TNF-α respectively.

2.14. Western blot

Protein concentration of cell lysates was determined with Bradford assay (Fermentas, Thermo Scientific, Waltham, MA, USA). Total protein (20 µg per lane) separated by 12% SDS-PAGE was transferred onto PVDF membranes (Merck, Darmstadt, Germany). After blocking (5% non-fat milk, 30 min), primary antibodies for p21, phospho (p)-H2A (Cell Signaling Technology, Inc.) were applied overnight at 4 °C, and anti-mouse IgG, HRP-linked Antibody or anti-rabbit IgG, HRP-linked antibody both at a dilution of 1:10.000, purchased from Jackson ImmunoResearch (West Baltimore Pike, West Grove, PA, USA) used for detection (2 h, RT). Reactive proteins were visualized using chemiluminescene reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Blots were probed with anti-PCNA antibody (Cell Signaling Technology, Inc.) for loading control.

2.15. RNA extraction, cDNA synthesis

RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed using Superscript III First Strand Synthesis Super Mix (Life Technology, Germany).

2.16. Real time PCR

qPCR was conducted with SYBR Green kit (Promega, USA) in a BioRad C1000 Thermal cycler/CFX96 real-time System (BioRad, Germany) for quantitative analysis of senescence markers. Briefly, 5 ng cDNA were added to a final volume of 10 µl/reaction containing 1 × SYBR Green PCR Master Mix (Promega, USA) and 100 nM of each respective primer. Thermal cycling conditions were: 95C 10 min, 40 cycles: 95 °C/30 s, 59 °C/ 30 s and 72 °C, 1 min for elongation. Primers are listed in Supplementary Table 2.

2.17. Statistical analysis

Statistical tests were performed with GraphPad Prism. For comparisons between two groups of normally distributed data with equal variance two-tailed unpaired t-test or one-way ANOVA (Dunett or Tukey) was used. For comparisons between two groups of nonparametric data two-tailed Mann-Whitney U test or Kruskal-Wallis test (Dunn) were executed to evaluate the statistical significance, which was considered at $p < 0.05$.

3. Results

3.1. Detection of PRAME-specific T-cell responses in four out of five healthy donors

Five healthy donors were recruited, four (donor1, 3, 4, and 5) showed more than 0.1% IFN-γ-secreting $CD4^+$ T cells after stimulation of their PBMCs with PRAME-peptide library and thus were subjected for expansion and enrichment of PRAME-specific T cells (Table 1). Frequencies of IFN-γ-secreting $CD4^+$ T cells varied between 0.1% and 5.48%. Interestingly $CD8^+$ T cells did not respond to PRAME peptides in donor1, 4 and 5 (Table 1).

3.2. Successful generation of PRAME-specific T cells in three individuals

PRAME-specific T cells were generated as described above from donor 1, 4 and 5 since these showed highest frequencies of IFN-γ-secreting $CD4^+$ T cells after stimulation with PRAME peptides. PRAME-specific T cells rapidly proliferated during 14 days: 815- to 9,411-fold expansion was achieved (Table 2). Expanded cells were mainly $CD4^+$ T cells with their share ranging from 62% to 92% (Table 2). Viability was always ≥ 96%.

3.3. PRAME-specific T cells differentiate into effector memory phenotype and secrete T_H1 cytokines upon ex-vivo culture

Immunological phenotype and cytokine profile of PRAME-specific T cells was verified with T-cell differentiation markers CD27, CD28, CD62L, CD45RO, and the secretion of IL-2, TNF-α and IFN-γ. The vast majority of PRAME-specific $CD4^+$ (IFN-γ-secreting) T cells displayed effector memory ($CD62L^-/CD45RO^+$) (97% ± 3.8%), a small proportion central memory phenotype ($CD62L^+/CD45RO^+$) (0.15% ± 0.17%), no naïve T cells ($CD27^+/CD28^+$) were found. Intracellular staining revealed IFN-γ (60.2% ± 5.4%), TNF-α (65.9% ± 4.4%) and IL-2 (32.6% ± 18.6%) secretion in expanded $CD4^+$ T cells in response to PRAME peptides compared to MOG-stimulation (Fig. 1A). More than 50% of $CD4^+$ T cells secreted both TNF-α and IFN-γ (56.9% ± 6.2%) (Fig. 1B) indicating a T_H1 profile for the generated PRAME-specific $CD4^+$ T cells.

3.4. IFN-γ and TNF-α in supernatants of PRAME-specific T cells is significantly increased by antigen-specific stimulation

Supernatants were analyzed with multiple cytokines assay LEGENDplex (BioLegend) and PRAME antigen-stimulated PRAME-specific T cell's supernatants compared with those from ***un***stimulated PRAME-specific T cells. As shown in Fig. 1C, IFN-γ, TNF-α, IL-2, IL-4, and sFasL were significantly increased in supernatants of PRAME-stimulated PRAME-specific T cells, with the combination of IFN-γ and TNF-α being of special interest since we and others have shown that combined T_H1 cytokines IFN-γ and TNF-α can drive cancer cells into senescence [10, 21].

Table 1
Cell frequencies ($CD3^+$, $CD4^+$ and $CD8^+$ T cells) from five healthy donors after 11 days of in vitro stimulation with PRAME overlapping peptides.

Donor	$CD3^+$ [%]	$CD4^+$ [%]	$CD8^+$ [%]	$CD4^+$ IFN^+ [%]	$CD8^+$ IFN^+ [%]
1	74.94	71.72	22.24	5.3	0.01
2	74.56	29.24	48.99	0.04	0
3	67.77	38.83	43.96	0.12	0.15
4	88.1	83.66	14.15	5.48	0.06
5	84.85	63.49	28.17	2.04	0

Table 2
Cell counts (viable cells) during the generation of PRAME-specific T cells (start, post-IFNγ enrichment and post expansion) in three independent runs.

Expansion	Start Cell count	Post-IFNγ Enrichment Cell count	Post Expansion Fold Expansion	Cell count	$CD3^+$ IFN^+ $CD4^+$ [%]	$CD3^+$ IFN^+ $CD8^+$ [%]
1	$1x10^9$	$4.7\ x10^6$	815	$3.8\ x10^9$	96	4
2	$1x10^9$	$1.7\ x10^6$	9411	$1.6\ x10^{10}$	91	9
3	$1x10^9$	$2.4\ x10^6$	1333	$3.3\ x10^9$	73	26

3.5. *Nomo-1 cells and Kasumi cells exhibit terminal growth arrest upon treatment with supernatants of antigen-stimulated PRAME-specific $CD4^+$ T-cell*

To investigate whether supernatants generated by individual antigen-stimulated PRAME-specific T cells can drive AML cells into terminal growth arrest, Nomo-1 and Kasumi cells were incubated with supernatants conditioned by antigen-stimulated PRAME-specific T cells for 96 h. Then cell-cycle phases of leukemic cells were evaluated. As shown in Fig. 1D, supernatants of antigen-stimulated PRAME-specific T cells arrested Nomo-1 cells in G1/G0 phase and massively reduced S-phase cells compared to the control (culture medium) or supernatants of antigen-***un***stimulated PRAME-specific T cells. Similar results were obtained with Kasumi cells (Fig. 1D).

3.6. *Nomo-1 cells and Kasumi cells remain in permanent cell-cycle arrest following incubation with combined T_H1 cytokines IFN-γ and TNF-α*

To access whether leukemic cells are permanently cell-cycle arrested through IFN-γ and TNF-α reseeding experiments were performed. After incubation **T_H1 cytokine**-containing medium was removed, AML cells were washed and regrowth allowed in fresh standard culture medium without any further additives. As seen in Fig. 2A, Nomo-1 and Kasumi-1 cells pre-exposed to IFN-γ and TNF-α were unable to regrow in any of the subsequent four passages, indicating that these cell lines entered a permanent cell-cycle arrest after treatment with IFN-γ and TNF-α.

3.7. *Morphologic and phenotypic changes of Nomo-1 and Kasumi cells after treatment with combined IFN-γ and TNF-α*

Kasumi and Nomo-1 cells treated 96 h with IFN-γ and TNF-α, showed significant morphological changes. Most remarkably Nomo-1 cells, normally growing in suspension, turned into adherent cell line with star-shaped extensions similar to dendritic cells (Fig. 2B). In addition, they significantly increased the expression of myeloid maturation markers CD11c, CD14, CD80, CD83 and also CD19, while Kasumi cells significantly upregulated CD11c, CD14, CD80, CD83, CD86, HLA-DR and additionally CD19 (Fig. 2C). These findings suggest that AML cells mature and gain DC-like phenotype and morphology after exposure to IFN-γ and TNF-α, indicating that growth-arrested cells can differentiate according to their original cell fate and gain lineage specific characteristics, as reported for IFN-γ/TNF-α growth-arrested rhabdomyosarcoma cells differentiating into bundles of cross-striated multinucleated muscle cells in-vivo [22].

3.8. *Senescent Nomo-1 and Kasumi cells significantly upregulate negative cell cycle regulator p21, and Kasumi cells additionally exhibit senescence marker pH2AX*

Senescence - the irreversible cell-cycle arrest in G1 or G2 phase - results in the accumulation of the cyclin dependent kinase (Cdk) inhibitors p21 (CDKN1A) and p16 (CDKN2A), which block the inactivating phosphorylation of the retinoblastoma tumor suppressor (pRb), thereby preventing DNA replication [23]. To determine whether cell-cycle arrested Kasumi and Nomo-1 cells after treatment with IFN-γ and TNF-α were "senescent" or not, several senescence markers were analyzed by qPCR and Western blot.

Medium supplemented with IFN-γ and TNF-α downregulated mRNA of cell cycle associated genes CDKN2A (p16), CHEK1, CHEK2, E2F1 and E2F3 but upregulated transcription for CCND1, CDKN1A (p21) and ETS1 in Nomo-1 cells and also increased CCNE1, CDK2, CDK4, CDKN2D, ETS2, TBX2 and TBX3 (Fig. 3A) mRNA compared to control. IFN-γ/TNF-α-exposed Kasumi cells downregulated transcription of cell cycle promoting genes: CCND1, CDK2, CDKN2A, CHEK1, CHEK2, E2F1, E2F3, TBX3 with the largest downregulation in E2F1 and TBX3 while p21, ETS1, and CCNE1 mRNA were strongly upregulated (Fig. 3A). E2Fs play a critical role in the control of cellular proliferation [24]. E2F1 and E2F3 were downregulated in both cell lines by combined T_H1 cytokines IFN-γ and TNF-α, indicating suppressed G1-phase progression [25]. In Nomo-1 cells CDKN2D was upregulated, a protein that has been shown to form a stable complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus functioning as a cell growth regulator controlling cell cycle by inhibiting G1 progression and G1/S transition [26].

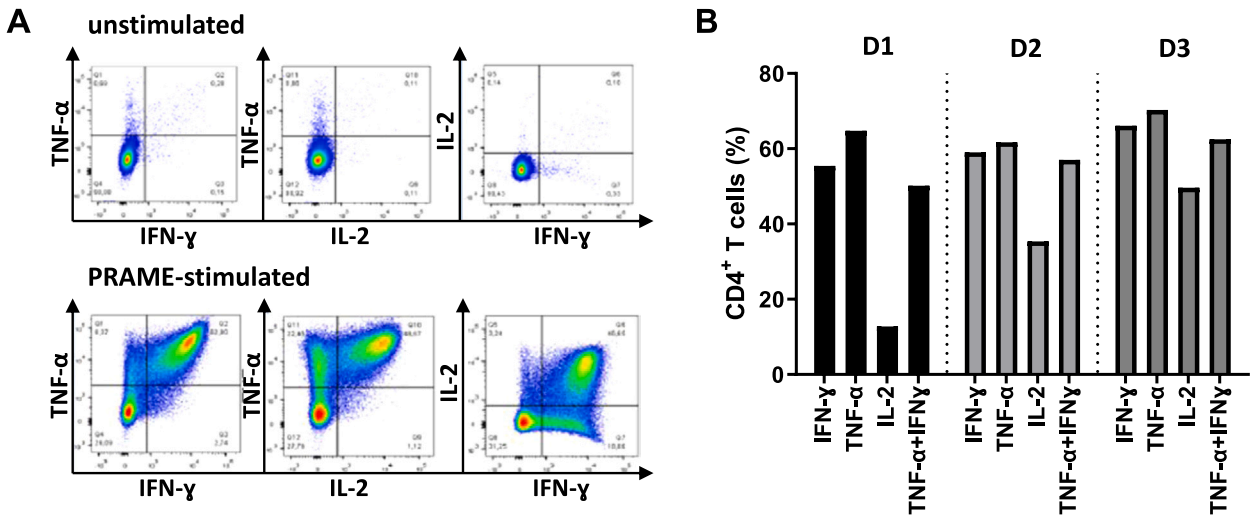
Upregulated protein expression was confirmed by Western blot for p21 in Nomo-1 and Kasumi treated with the combined T_H1 cytokines IFN-γ and TNF-α (Fig. 3B). In addition, Kasumi displayed increased phosphorylated histone H2AX (pH2AX) after exposure to supernatants of antigen-stimulated PRAME-specific T cells (Fig. 3C), the most sensitive epigenetic marker of double-stranded DNA breaks (DSBs) and telomere shortening which are both increased in damaged and senescent cells [27]. These results strongly suggest that combined T_H1 cytokines IFN-γ and TNF-α induce senescence in Nomo-1 and Kasumi cells through the *p21* cascade and that senescence induction in Kasumi cells also involves pH2AX.

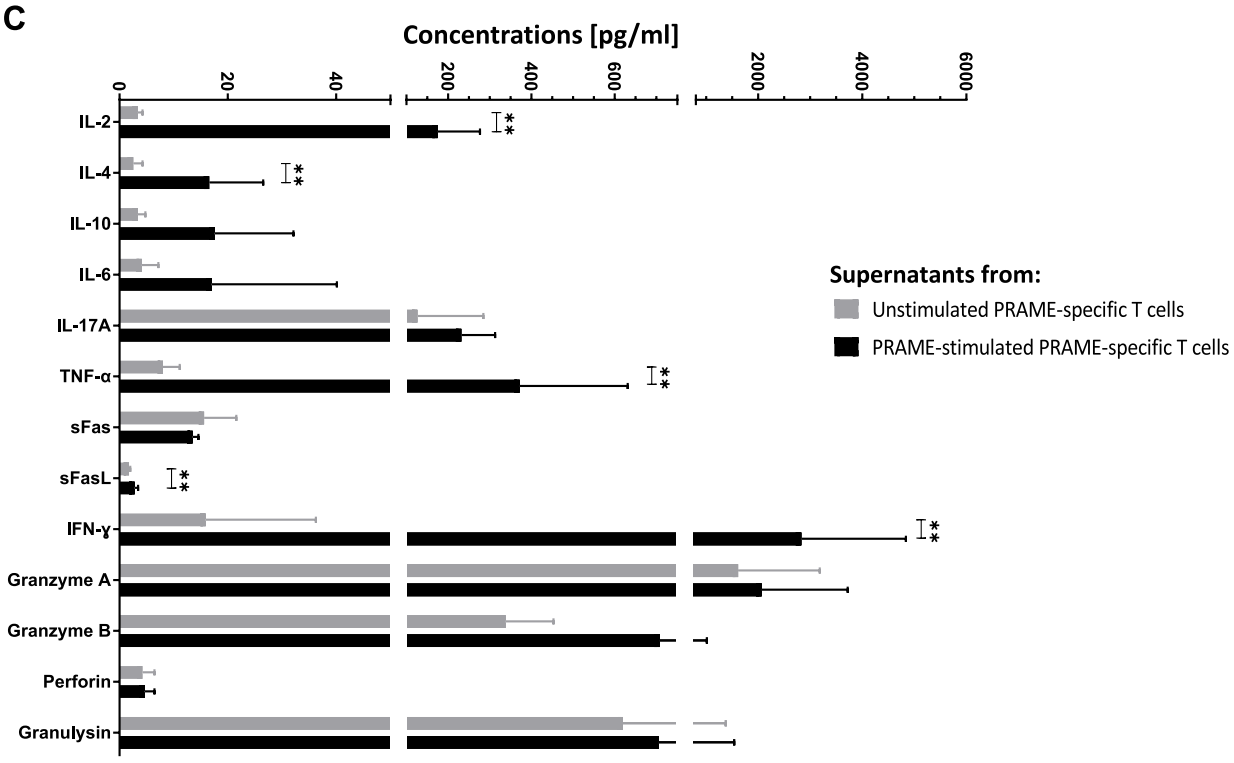
3.9. *Chemokines and cytokines secreted by Nomo-1 and Kasumi cells display a SASP after incubation with supernatants of antigen-specific stimulated PRAME-specific T cells or recombinant T_H1 cytokines IFN-γ and TNF-α*

Nomo-1 and Kasumi cells were analyzed for the secretion of chemokines after incubation with TNF-α (20 ng/ml) and IFN-γ (100 ng/ml) (Fig. 4A) and after treatment with supernatants of *un*stimulated or antigen-specific stimulated PRAME-specific $CD4^+$ T cells.

Senescence-associated chemokines - excluding GROa, ENA-78, IP-10 and TARC - were detected in significant amounts compared to control in medium conditioned by Nomo-1 after exposure to supernatants from PRAME-stimulated PRAME-specific T cells (Fig. 4B). Kasumi cells showed a statistically significant increase in IP-10, Eotaxin, MCP-1, RANTES, MIP-1a, MIG, MIP-3α, I-TAC and MIP-1β after treatment with supernatants from antigen-stimulated PRAME-specific T-cells (Fig. 4B). These results affirm that antigen-stimulated PRAME-specific $CD4^+$ T cells can induce SASP in Nomo-1 and Kasumi cells.

Chemokines released by NOMO-1 and Kasumi cells in response to recombinant IFN-γ and TNF-α slightly differ from those released after stimulation with PRAME-stimulated -specific T cell supernatants. This is reasonable since the supernatant contains diverse immune populations sectretome after a mutual crosstalk condition, thus can further modulate the secretome of AML cells. Slight differences between the secretome of NOMO-1 (AML FAB M5a) and that of Kasumi (AML FAB M2) in response to the *same* stimulus very likely reflect the respective differentiation status of the leukemic cell lines. However, the highly significant increases of cytokines and chemokines characterizing a SASP [28] qualifies the statement that in both cell lines the secretome belongs to senescent cells after stimulation with recombinant or supernatant-derived T_H1-cytokines.





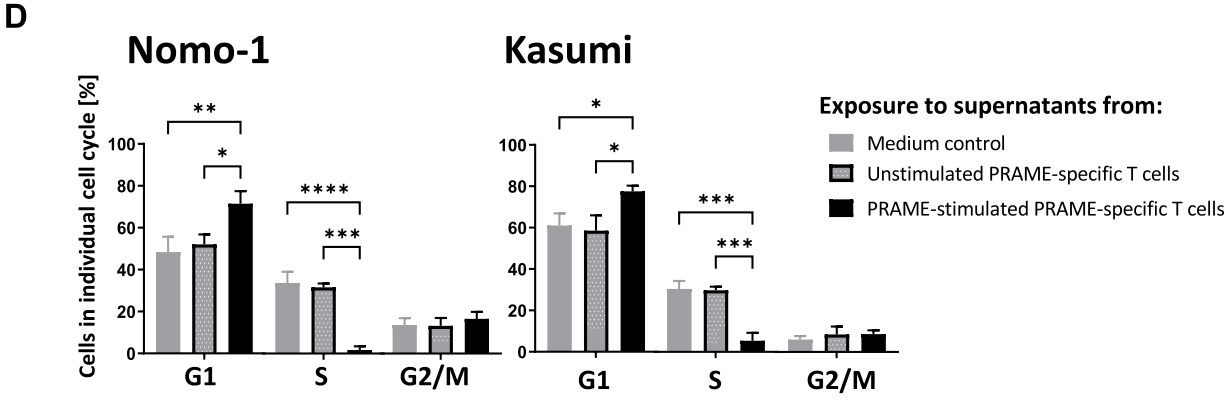


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Fig. 1. PRAME-specific T cells differentiate into effector memory phenotype and secrete T_H 1 cytokines. (A) Representative flow cytometric plots of IFN-γ^+, TNF-α^+ and IL-2^+ PRAME-specific $CD4^+$ T cells in response to PRAME peptide library. (B) Frequency of IFN-γ^+, TNF-α^+ and IL-2^+ PRAME-specific $CD4^+$ T cells is given for 3 donors (D1-D3) separately (C) Cytokine concentrations in supernatants conditioned by PRAME-peptides-stimulated and -unstimulated PRAME-specific $CD4^+$ T cells. Bars represent the mean ± SD of 3 independent experiments with supernatants from 3 different donors (n = 3) Data were analyzed with two-tailed Mann-Whitney U test. (D) Nomo-1 and Kasumi cells exhibit growth arrest upon treatment with supernatants of antigen-stimulated PRAME-specific $CD4^+$ T-cells. Percentages of Nomo-1 and Kasumi cells in different cell-cycle phases after 96 h incubation with supernatants from antigen-stimulated PRAME-specific $CD4^+$ T cells. Shown are mean values with SD of three independent experiments (n = 3). Significances were tested with one-way ANOVA (Tukey`s). Statistical significance: * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

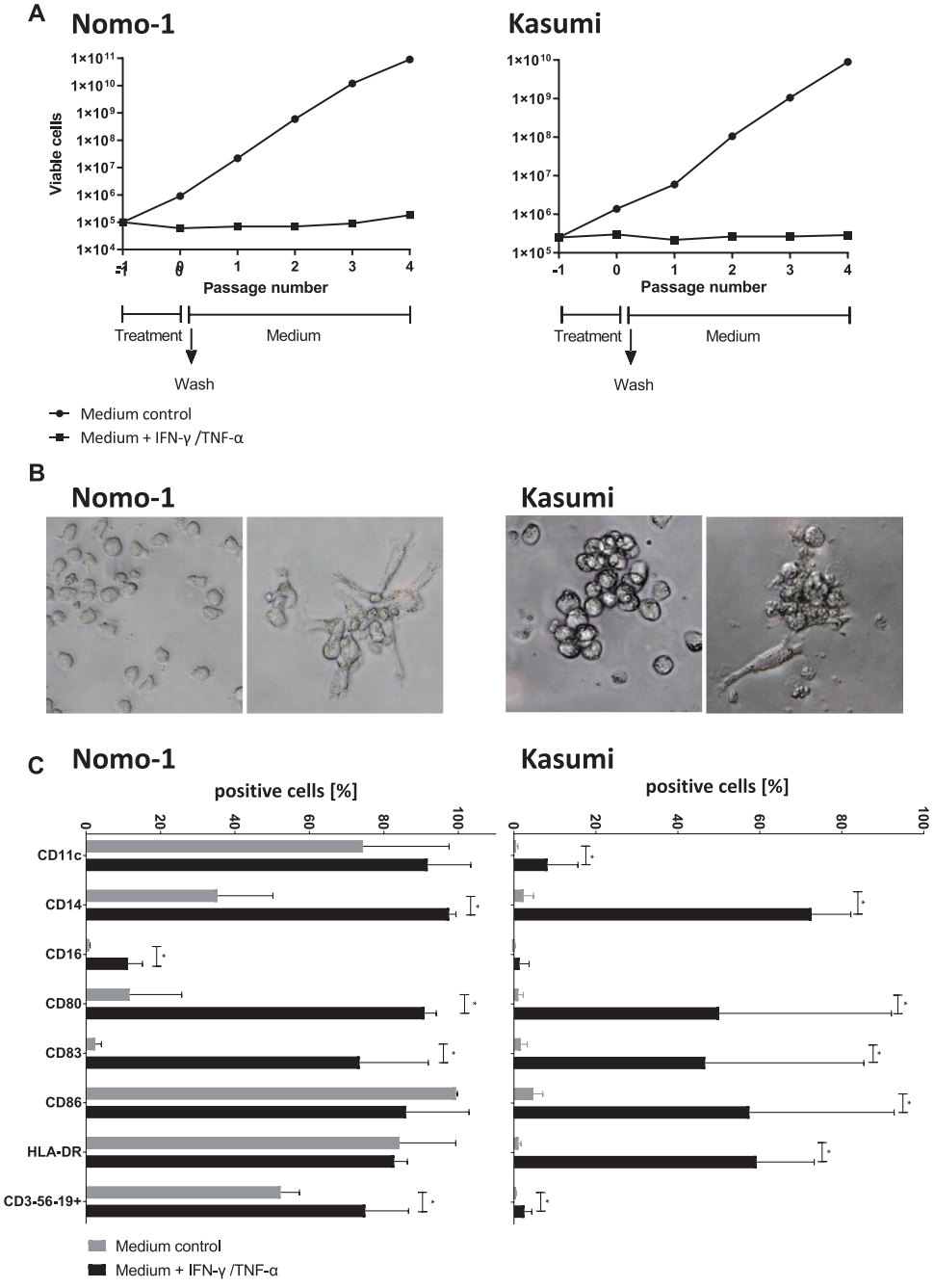

Fig. 2. Reseeded Nomo-1 and Kasumi cells do not proliferate after incubation with TNF-α and IFN-γ (A) Proliferation curves of Nomo-1 and Kasumi cells pre-incubated with IFN-γ (100 ng/ml) and TNF-α (20 ng/ml). After 96 h of exposure, cells were washed, seeded in new plates and cultivated for a total of 4 passages analogous to untreated control. Data representative of four independent experiments with consistent results (n = 4) are shown for each cell line. (B) Nomo-1 and Kasumi cells gain DC-like morphology after treatment with combined IFN-γ^+ and TNF-α^+. Nomo-1 and Kasumi cells incubated with medium (a and c) or medium plus IFN-γ^+/TNF-α^+ (b and d) for 96 h. Representative pictures of two independent experiments with same results (n = 2) are shown. Pictures were taken with Canon EOS 550D and a Zeiss Anxiovert 135 microscope. (C) AML cells express myeloid maturation markers after exposure to combined IFN-γ^+ and TNF-α^+. Data were analyzed with two-tailed Mann-Whitney U test. Statistical significance: * $p < 0.05$.

3.10. Senescence in Nomo-1 and Kasumi cells induced by supernatants of antigen-stimulated PRAME-specific T cells is caused by the combination of IFN-γ with TNF-α

Nomo-1 and Kasumi cells were incubated with the supernatants of antigen-stimulated PRAME-specific T cells in the presence or absence of neutralizing anti-IFN-γ and/or anti-TNF-α antibody. As shown in Fig. 5A, single IFN-γ- or TNF-α-neutralization reduced the magnitude of cell-cycle arrest, while simultaneous neutralization of IFN-γ- and TNF-α restored cell-cycle progression. These findings unequivocally indicate that senescence induction in Kasumi and Nomo-1 cells after exposure to supernatants of antigen-stimulated PRAME-specific T cells is mediated

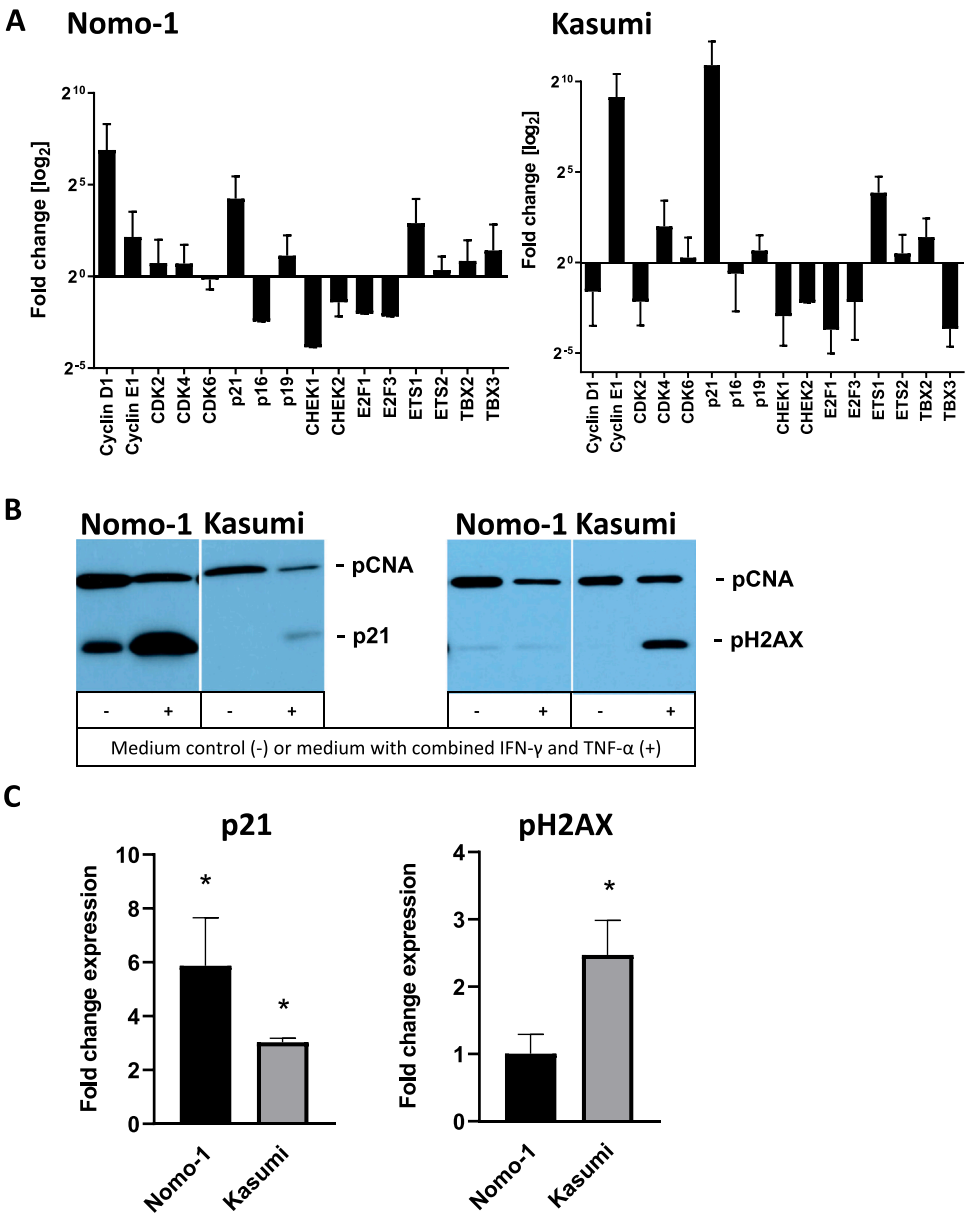

Fig. 3. p21 is upregulated in both Nomo-1 and Kasumi-1 cells, and epigenetic senescence marker pH2AX additionally upregulated in Kasumi-1. (A) senescence markers in Nomo-1 and Kasumi cells after 96 h treatment with combined IFN-γ^+ and TNF-α^+ analyzed by qPCR compared to untreated control. Bars represent mean ± SD of three independent experiments (n = 3). (B) Protein expression of p21, pH2AX and house-keeping gene pCNA are shown for Nomo-1 and Kasumi cells after 96 h exposure to combined IFN-γ^+ and TNF-α^+. Representative Western blots of four independent experiments for p21 in Nomo-1 (n = 4) and Kasumi (n = 2) and representative blots of three independent experiments in Nomo-1 (n = 3) and Kasumi (n = 3) with consistent results are shown. Nomo-1 and Kasumi samples shown in the Western blot were run on the same gel, relevant blots for p21 and pH2AX were assembled. (C) Given is the average fold change of p21 and pH2AX expression with respect to untreated control for the experiment shown in (B). Data were analyzed with two-tailed Mann-Whitney U test. Statistical significance: * $p < 0.05$.

by the combination of T_H1 cytokines IFN-γ and TNF-α.

3.11. T_H1 cytokines induce senescence in fresh patient-derived AML blasts

AML M5 blasts of a 7-month-old patient at day of diagnosis were incubated in medium supplemented with T_H1 cytokines either single or combined as well as in supernatants of Zoledronate-stimulated or αCD3/αCD28-stimulated PBMCs from a healthy donor. Cell-cycle phases and p21 and p16 mRNA level were analyzed. As shown in Fig. 5B, IFN-γ, combined IFN-γ/TNF-α, supernatants of Zoledronate-stimulated or αCD3/αCD28 -stimulated PBMCs all changed cell-cycle compartment: reducing cells in S-phase while at the same time increasing cells in G1-phase. Correspondingly p21 was highly upregulated (22.5-fold) by IFN-γ, combinative T_H1 cytokines (20.35-fold), supernatants of Zoledronate-stimulated PBMCs and elevated expression of p16 was found after single IFN-γ and combinative T_H1 cytokines (Fig. 5C). Despite a single case analysis, these findings suggest that T_H1 cytokines are involved in senescence induction in AML cells in-vivo. Regarding the finding that TNF-α didn't change the cell cycle of AML patient primary cells, it can be speculated that this escape might be due to TNFR mutations or downstream signaling pathway components as we have shown this mechanism of escape for senescence-resistant *Tnfr1*$^{-/-}$ RIP-Tag2 β-cancer cells in our previous study [10].

3.12. Supernatants of CMVpp65 peptide responsive CMV-specific T cells and of TCR-activated Vδ2$^+$ T cells drive AML cells in permanent cell-cycle arrest

Given that the combination of IFN-γ and TNF-α suffices for cell-cycle arrest induction in AML, we hypothesized that supernatants of antigen-stimulated virus-specific or even those of self-derived antigen-specific T cells (Vδ2$^+$ T cells) can also induce senescence in AML. Therefore, we generated CMV-specific T cells as described and investigated whether supernatants of pp65-stimulated CMV-specific T cells can also induce senescence in Nomo-1 and Kasumi cells. We also tested supernatants from zoledronic acid-stimulated PBMCs i.e. cell-culture derived cytokines from TCR-activated Vδ2 T cells. Heatmap of the cytokines shows that the supernatants of these cells all contained IFN-γ and TNF-α (Supplementary Figure 1). Both, supernatants of CMV-specific T cells

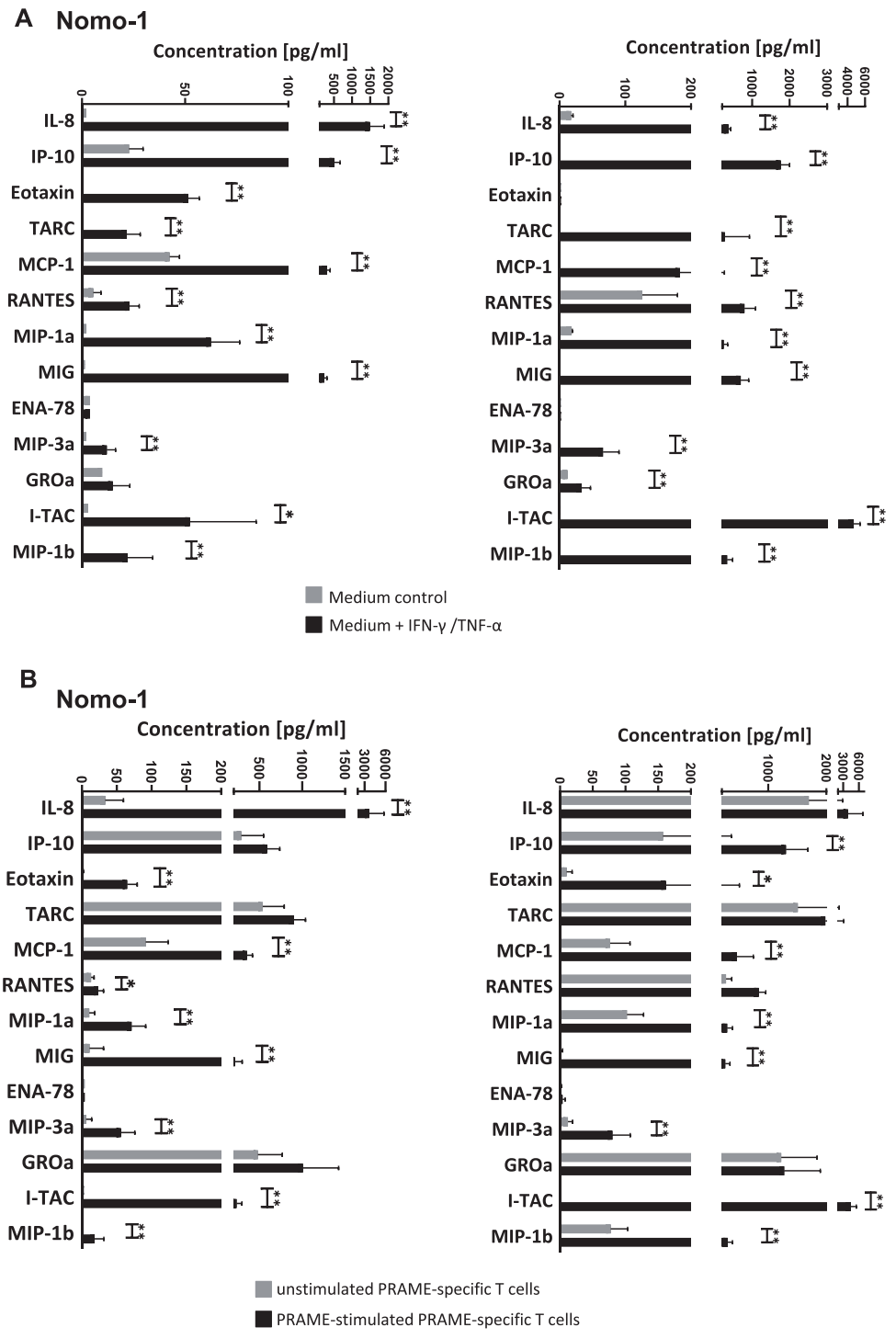

Fig. 4. Nomo-1 cells and Kasumi cells generate SASP after treatment with supernatants of antigen-stimulated PRAME-specific T cells. Chemokines (pg/ml) in medium conditioned by Nomo-1 or Kasumi cells after treatment with (A) 20 ng/ml TNF-α and 100 ng/ml IFN-γ compared to control, (B) supernatants of *un*stimulated PRAME-specific $CD4^+$ T cells and antigen-stimulated PRAME-specific $CD4^+$ T cells were examined with a multiplex assay. Bars represent the mean ± SD of three independent experiments after 96 h of treatment, respectively. Data were analyzed with two-tailed Mann-Whitney U test. Statistical significance: * $p < 0.05$, * * $p < 0.01$.

and that of TCR-activated $V\delta2^+$ T cells induced cell-cycle arrest (Fig. 6A) and upregulated p21 (Fig. 6B).

4. Discussion

In the present study, we demonstrate that supernatants of antigen-stimulated (PRAME)-specific T cells induce senescence in Kasumi and Nomo-1 cells. We then prove combined IFN-γ and TNF-α, produced by antigen-stimulated PRAME-specific $CD4^+$ T cells as causative agent for this event. To our knowledge this is the first study to show that i) senescence is induced in AML cells by T_H1 cytokines IFN-γ and TNF-α, and ii) senescence is induced in AML cells by cytokines derived from TCR-engineered $CD4^+$ T cells. However, even more important is the fact, that iii) T-cell responses directed specifically towards an antigen that is NOT expressed by leukemic tumor cells can induce terminal growth arrest in malignant hematopoietic cells as we show senescence induction in AML cells by cytokines originating from TCR-activated $V\delta2^+$ T cells or CMV-specific T cells (graphical abstract).

Efficacy of PRAME-specific T cells is under investigation in clinical trials targeting hematopoietic malignancies expressing PRAME (NCT02743611, and NCT03503968). However, senescence-inducing activity of PRAME-specific T cells has not been systematically studied. Chronic inflammation mediated by a SASP can lead to residual leukemic cells and disease recurrence [5,6] while SASP is also supposed to inhibit

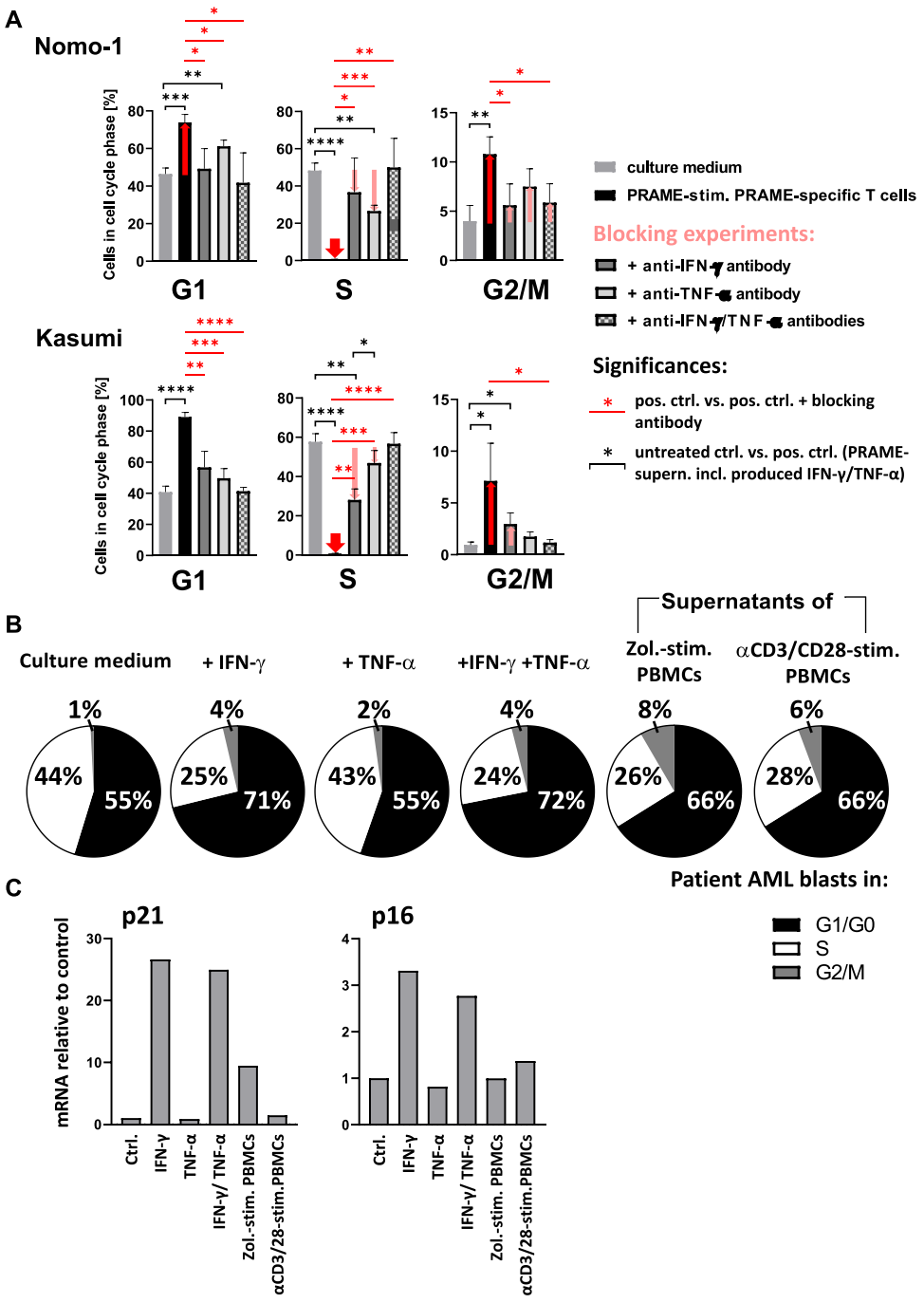

Fig. 5. Cell-cycle analysis shows that senescence is induced in Kasumi and Nomo-1 cells by combined action of IFN-γ and TNF-α by supernatants of antigen-stimulated PRAME-specific T cells. (A) Cell-cycle analysis was conducted by EdU assay using Nomo-1 cells and Kasumi cells incubated with supernatants of antigen-stimulated PRAME-specific $CD4^+$ T cells in the presence or absence of anti-IFN-γ neutralizing antibody and or anti-TNF-α neutralizing antibody. Bars represent the mean ± SD of three independent experiments (n = 3). Data were analyzed with two-tailed unpaired t-test. Statistical significance: * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * *** * $p < 0.0001$. (B and C) Senescence is induced in primary AML patient blasts by combined T_H1 cytokines as well as supernatants conditioned by Zoledronate-stimulated or αCD3/αCD28-stimulated PBMCs. (B) Cell-cycle phases of T_H1 cytokine- or supernatant-treated patient AML blast cells after 96 h exposure are shown, with supernatants conditioned by either Zoledronate-stimulated or αCD3/αCD28-stimulated PBMC. (C) Corresponding mRNA level of p16 and p21 with fold change gene expression compared to untreated control are given for each treatment.

the proliferation of cancer cells and stimulate immunosurveillance which eliminates malignant cells [7,8]. Although the impact of senescent AML cells on the disease outcome is still controversial, it is warranted to assess the overall effect of senescence-inducing activity by PRAME-specific T cells in future clinical trials.

In our assay TNF-α and IFN-γ released by PRAME-antigen-stimulated PRAME-specific T cells was 0.37 ± 0.26 ng/ml and 2.83 ± 2.01 ng/ml, respectively. Intriguingly, these values are significantly i.e. about 200-fold lower than the ones implemented in our previous studies (20 ng/ml TNF-α and 100 ng/ml IFN-γ) for senescence induction in solid malignancies [10,22,29]. Whether there is a discrepancy in sensitivity to senescence inducing TNF-α and IFN-γ is an interesting question and remains to be shown as well as the minimum amount needed to induce senescence. The very small amounts of TNF-α and IFN-γ that we need in AML for the induction of senescence suggests that this is a truly physiologic mechanism contributing to control infected, stressed and/or malignant/transformed cells and that this might especially work in AML patients. The concentration of 20 ng/ml TNF-α and 100 ng/ml IFN-γ, however, correspond to levels detected in conditioned media of CMV-peptide/EBV-peptide-stimulated T cells in PBMC cultures of CMV- or EBV-positive donors (given in Supplementary Figure 1).

Since we intend to show the finding that independent from their specificity T cells can induce senescence in leukemic cells, we worked with low but also high concentrations of T_H1 cytokines, the latter secreted by T cells responding to neoepitopes. The significant difference to TNF-α and IFN-γ secreted by PRAME-specific T cells after stimulation with PRAME-peptide and those produced by CMV-specific or EBV-specific T cells in response to their cognate antigens may result from

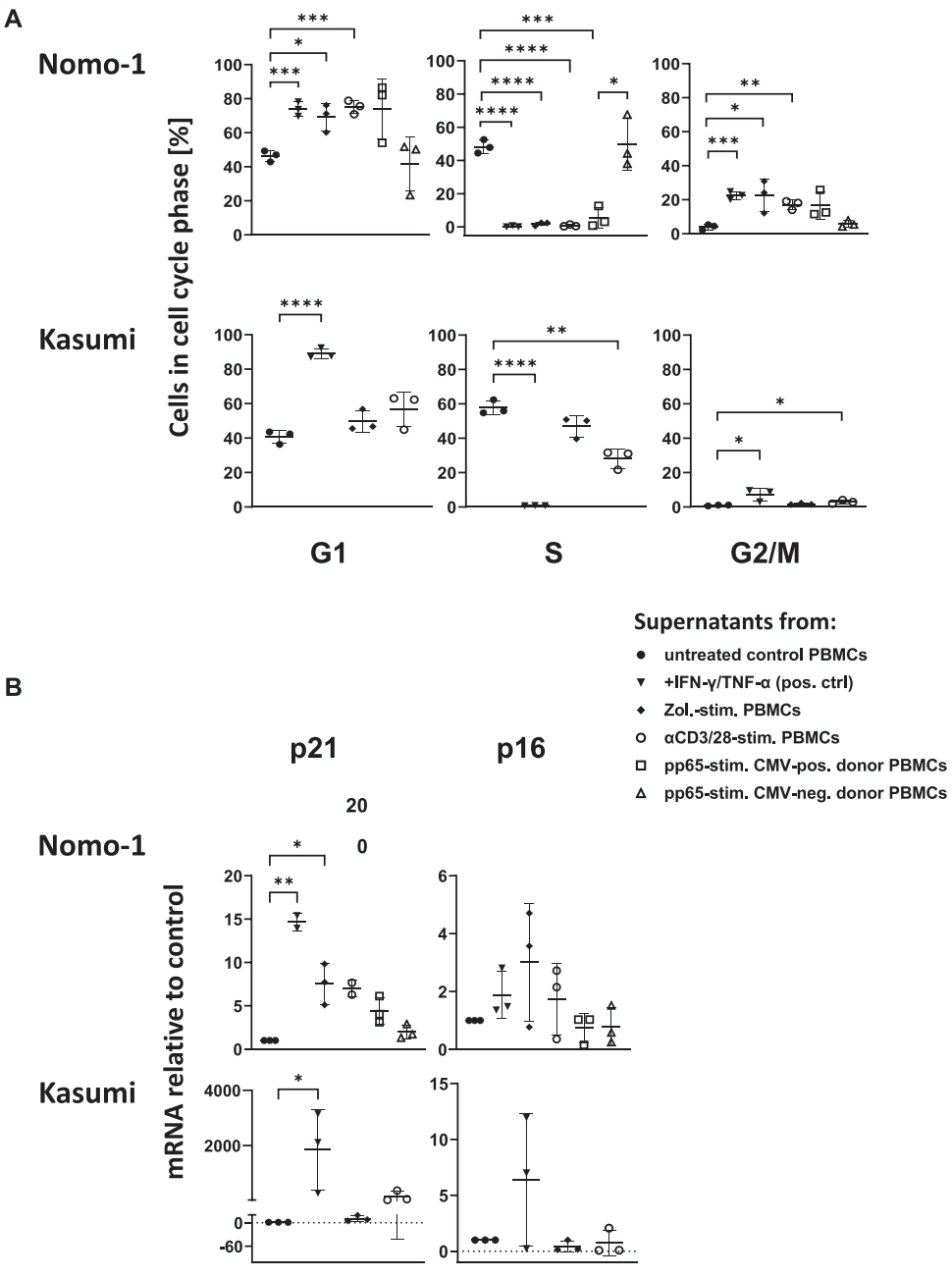

Fig. 6. T_H1 cytokines secreted by CMV Peptide-stimulated CMV-specific T cells can drive AML cells in terminal growth arrest. (A) Percentages of Nomo-1 and Kasumi cells in different cell-cycle phases after a 96 h exposure to supernatants from PBMCs exposed to diverse stimuli. Given is the mean value with SD of three independent experiments (n = 3). Significances are given only for treatments in relation to untreated control. Two-tailed unpaired t-test was used for statistical analysis. Shown are the mean values with SD with respect to untreated control cells (n = 3). (B) T_H1 cytokines secreted by CMV Peptide-stimulated CMV-specific T cells can drive AML cells in terminal cell-cycle arrest, which is accompanied by the induction of cell cycle inhibitory p21 and p16. The expression of p21 and p16 was analyzed by qPCR. Kruskal-Wallis test with Dunn's multiple comparisons was used for statistical analysis. Shown are the mean values with SD with respect to untreated control (n = 3). * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

the fact that PRAME constitutes a self-peptide against which the TCR repertoire has been selected in the thymus. In contrast, viral pp65- and BZLF1-derived peptides are neoepitopes, recognized by an unselected T cell pool, harboring TCRs with high affinity for these antigens, with TCR signal strength controlling cytokine production [30]. Thus, in this study we show with cell-cycle analysis that NOMO-1 and Kasumi get G1/0 growth arrested by supernatants from self-peptide-reactive T cells containing low level TNF-α and IFN-γ (Fig. 5A), by recombinant TNF-α and IFN-γ (high level) (Fig. 6), and also by supernatants derived from Zoledronate-stimulated, αCD3/28-stimulated or pp65-stim. CMV-pos. donor PBMCs, but not from pp65-stim. CMV-negative donor PBMCs or medium alone. Thus, this study demonstrates sensitivity and effectiveness for senescence induction by TNF-α and IFN-γ within physiologic concentrations in a range over more than 2 log scales.

Furthermore, we show that neutralization of single IFN-γ or TNF-α reduces the magnitude, while simultaneous neutralization completely abolishes the cell-cycle arrest effect (Fig. 5A). Thus, a complete growth arrest is achieved only by the combination IFN-γ plus TNF-α. This finding is compatible with previous reports that combined TNF-α and IFN-γ induce senescence to a maximum extent and either cytokine alone cannot comparably [10,22,29].

Although the precise mechanism is still not described and the occurrence is rare, complete and partial remissions have been repeatedly reported after severe systemic infections [31,32] and sepsis [33,34], since Eisenlohr in 1878 reported the first case of regression of "marked leukocytosis" after a severe typhoid infection [35]. In addition to Rashidi and Fisher's review article reporting 46 cases of "spontaneous remission" of AML from 1951 to 2015 [36], more have been described recently [33,37–40]. The majority of patients (91.3%) described in the Rashidi and Fisher study had fever prior to spontaneous remission and 32 patients (71.1%) suffered from a documented infection with the most common infections such as pneumonia, bacteremia, and skin/soft tissue infections [36]. In a most recent report [41] excessive activation and production of the pro-inflammatory cytokines TNF-α and interleukin 2

(IL-2) (IFN-γ was not investigated) was suggested as a leading force for an anti-leukemia effect and as a potential mechanism that contributes to remission of leukemia. All of these events are accompanied by the increased activity of T lymphocytes, macrophages, and natural killer (NK) cells and specific and extensive T_H1 cytokine release comprising both TNF and IFN [41]. Elevated serum TNF-α and IL-2 have been reported for a patient with pneumonia, and an increased activity of NK cells, during active infections in patients with spontaneous remission of AML [42,43] with NK cells as a potent source of IFN-γ and TNF-α [44,45]. Moreover a negative correlation was also established 30 years ago between Tumor necrosis factor and RNA expression of hematopoietic growth factor genes in fresh blasts from patients with acute myeloid leukemia [46] and also B-ALL [47]. In conclusion, based on their and our findings, it can be speculated that systemic infections - triggering NK and pathogen-specific T-cell expansion and prominent cytokine release - can induce senescence/remission in AML cells [10]. Spontaneous clinical anti-leukemia response including transient and complete remissions in context with immune effector activation and cytokine production is strong evidence for a significant role that the patient's immune system can have in AML. Our report offers a new and exciting conceptual framework for the exploration of potent and novel immunotherapeutic approaches in the treatment of AML by focusing on the biological potential of T_H1 cytokines derived from activated T_H1 immune effector cells.

CRediT authorship contribution statement

NK, JS, HH and DG analyzed senescence induction in AML cell lines and patient blasts by supernatants from $V\delta2^+$ T cells, generated aCD3/aCD28-stimulated or CMV-specific T cells, performed cell cycle studies and qPCR and prepared the figures; LD analyzed senescence induction in AML cell lines after TNF/IFN-γ treatment, examined proliferation, morphology and phenotype, senescence marker expression (qPCR and Westernblot), senescence-associated secretory phenotype (SASP), generated supernatants from PRAME-specific T_H1 cells and analyzed their composition; ECM generated PRAME-specific $CD4^+$ T cells; MS and SS supervised experimental work and analyzed data regarding PRAME-specific T cells, HH analyzed the data and wrote the manuscript, KS conceptualization of the study, interpretation of data, wrote and critically revised the manuscript.

Statement conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.leukres.2022.106842.

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