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Original Research

Kv11.1-dependent senescence activates a lethal immune response via tumor necrosis factor alpha

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ABSTRACT

Understanding the complex relationship between cancer and immune surveillance is essential for leveraging the immune system to control tumor growth. In our study, we discovered that activating the Kv11.1 potassium channel in ER+ breast cancer cells induces a senescent phenotype, which in turn triggers a potent immune response against these senescent cells. Specifically, we found that the senescence-associated secretory phenotype (SASP) plays a crucial role in activating CD4+ *T*-helper 1 (Th1) cells and memory T cell phenotypes. This activation led to the release of tumor necrosis factor-alpha (TNFα), which induced the death of senescent breast cancer cells, independent of their resistance to endocrine therapy. Our findings suggest that Kv11.1 channel-induced cellular senescence in ER+ breast cancer cells is a key mechanism in immune surveillance, driving a lethal immune response through TNFα. These results highlight the potential immunomodulatory role of Kv11.1 activation in ER-positive breast cancer and provide a foundation for future therapeutic investigations.

Introduction

Breast cancer (BC) stands as the most prevalent malignancy and a leading cause of cancer-related deaths among women globally [1]. BC represents a heterogeneous class of diseases, each treated based on specific molecular signatures. Estrogen receptor (ER)-positive breast cancers typically undergo treatment with selective ER modulators (SERMs) like tamoxifen, followed by selective ER degraders (SERDs) such as fulvestrant in cases of SERM resistance [2]. These treatments generally yield a better prognosis compared to hormone receptor-negative BC. However, resistance to SERMs/SERDs and limited alternative therapeutic strategies are major contributors to cancer recurrence [3].

Bioelectricity plays a fundamental role in controlling a cellular homeostasis in both physiological and pathological conditions including cancer. The KCNH2 gene typically encodes the voltage-gated potassium ion channel Kv11.1 in highly differentiated cells of the brain, colon, and heart [4]. However, Kv11.1 expression has been identified in cancer cells of various histogenesis, including all types of BC cells [4–6]. The role of the Kv11.1 channel in cancer biology remains controversial, exhibiting both pro-tumorigenic and onco-suppressive behaviors, often dependent on the cancer context [6]. Previous research in our lab has shown that pharmacological stimulation of Kv11.1 in BCs inhibits tumor growth by inducing a senescent phenotype in cancer cells [7–10].

Cellular senescence can be triggered by a variety of stimuli that can affect different mechanisms resulting in an heterogenous and complex phenotype [11–13]. Generally, the senescent phenotype is marked by stable cell cycle arrest with high expression of tumor suppressors such as p21, the tumor necrosis factor alpha (TNFα) receptor 1 (TNFαR1) and by the Senescence-Activated Secretory Pathway (SASP) [14] which consists of secretion of a plethora of immunomodulators in the tissue microenvironment. While senescence is a long-lasting powerful mechanism to prevent tumor growth and is exploited as a therapeutic approach against cancer [15], there is controversial evidence about the role of SASP in controlling the immune response against cancer and in chemotherapy resistance [16–18]. For example, SASP from p53- wild-type cancer cells can promote polarization of immune system cells which eventually become involved in the tumor clearance [19]. In contrast, p53-null

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cancer cells produce a SASP that suppresses the immune response and/or promotes drug resistance. Furthermore, different senescent inducers and cell types can determine the quantity and quality of the SASP [20]. However, the identity of the specific immune cells that mediate these processes is not clear.

The immune system operates through a complex network of soluble factors, such as cytokines and proteins, which coordinate responses across various cell types. Immune surveillance plays a crucial role in controlling tumor growth. Both innate immune cells, like natural killer (NK) cells, and adaptive immune cells, including CD4+ and CD8+ *T* cells and B cells, work together to eliminate tumor cells. Traditionally, CD4+ *T* cells differentiate into distinct T helper (Th) subsets upon exposure to cytokines or antigens (e.g., MHC-II-restricted epitopes), producing tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), which assist CD8+ *T* cells in exerting cytotoxic effects on cancer cells. However, under certain conditions, such as self-antigen production, CD4+ *T* cells can exhibit direct cytotoxicity against tumor cells, highlighting a CD8-independent immune response against cancer. Recently, the therapeutic concept that chimeric antigen receptor (CAR) T cells that target senescent cells can be effective senolytic agents has been tested demonstrating that this approach could be considered as a successful therapeutic approach [15].

Despite the immune system's efforts, cancer can evade immune surveillance, a hallmark of tumor progression. TNFα, a multifunctional cytokine, plays a dual role in this context. On one hand, TNFα can induce cell death through activation of TNFα receptor 1 (TNFαR1), while on the other hand, TNFα receptor 2 (TNFαR2) promotes cell survival via nuclear factor κB (NF-κB) signaling. These opposing functions underscore the need to carefully assess TNFα-targeted anticancer therapies. Although advancements in immunotherapy have improved cancer treatment, breast cancer (BC) remains poorly immunogenic, posing a challenge for effective immune-based therapies.

Unfortunately, the well-known absence of an ER-positive cell line for syngeneic studies restricts our research. Nevertheless, this study, we present evidence that Kv11.1 activity-dependent senescence-associated secretory phenotype (SASP) in ER+ breast cancer cells drive the polarization of Th1 cells, resulting in TNFα-mediated death of senescent cancer cells. This represents a novel mechanism of immune response in ER+ breast cancer that is independent of CD8+ *T* cells and NK cells.

Results

Enrichment analysis of Differentially Expressed Genes (DEGs)

We conducted a Gene Ontology (GO) term enrichment analysis on the estrogen receptor positive (ER-positive) MCF7 breast cancer (BC) cells which exhibited a senescent phenotype upon activation of the Kv11.1 potassium channel with NS1643 (REF). We analyzed differentially expressed genes (DEG) based on biological processes (BP), then performed KEGG and REACTOME pathway enrichment analyses (Fig. 1A). Notably, more than 38% of both upregulated and downregulated genes belonged to the immune system category, representing the highest percentage among all gene sets (Fig. 1B). Remarkably, the upregulated genes were predominantly enriched in the immune system process (Fig. 1C), while the downregulated genes showed enrichment in the control of the cell cycle (Fig. 1D). Furthermore, we conducted an interrelation analysis focusing on the immune system process of upregulated DEG. This investigation revealed a significant enrichment of upregulated genes in the regulation of T cells (Fig. 1E). These findings suggest that the activation of the Kv11.1 channel by NS1643 in BC cells induces an immune response that is mediated by T-cells.

Kv11.1 activation induce SASP in ER-positive breast cancer cells

Based on our in-silico data, we wanted to test the hypothesis that activation of a Kv11.1-dependent senescence in BC cells produces an effective immune response. We expanded our previous investigation on the effects of Kv11.1 activation by monitoring cellular senescence markers such as p21, β-galactosidase and SASP markers in a panel of naïve or SERM/SERD resistant BC cells. The cell lines included in our tests were naïve ER-positive (MCF7, T47D), fulvestrant resistant T47D ($T47D^{Ful}$), ER-negative (MDA-MB-231) BC cell lines. Because the somatic point mutation D538G in the estrogen receptor alpha (ERα; $ERα^{D538G}$) is the most prevalent in acquired resistance to endocrine therapy, we generated a MCF7 cells line that constitutively expresses $ERα^{D538G}$ ($MCF7^{D538G}$; supplementary Fig. 1A). We found that cells that were treated with the Kv11.1 channel activator NS1643 (50μM/24hr) exhibited a significant increase in p21 (Fig. 2A) and β-galactosidase (Fig. 2B) regardless of ER expression or SERM/SERD resistance. In contrast, while NS1643 increased SASP in ER-positive cells (Fig. 2C; MCF7; T47D), no significant changes in these senescence parameters were observed in the ER-negative BC cells (Fig. 3C; MDA-MB-231). Additionally, no changes in any senescent markers were observed in the non-carcinogenic MCF10A breast cells treated with NS1643 (Fig. 2A-C). These data indicate that stimulation of the Kv11.1 potassium channel induces a senescent phenotype specifically in BC cells but fails to produce SASP in the ER-negative subtype.

Kv11.1-dependent SASP activates a lethal immune response

The role of secreted molecules from senescent cells in controlling the immune response is still controversial. We investigated the impact of the Kv11.1-associated senescence-messaging secretome (SMS) on the immune response. We exposed peripheral blood mononuclear cells (PBMC) to the media collected from BCSen (referred to as SMS-medium; Fig. 3A). After 24 h treatment we collected separately both PBMC cells (primed-PBMC) and the medium in which they were bathed (PBMC-conditioned medium; PBMCcm). Subsequently, we exposed distinct groups of naïve BCSen cell lines to the primed-PBMC or PBMCcm (Fig. 3B-D) for 24-h and performed a killing assay via Annexin V/PI flow cytometry.

We found that primed-PBMC or PBMCcm produced a significantly higher death rate in senescent ER-positive MCF7 (Fig. 3B-D), $MCF7D^{538G}$ (Fig. 4A-C), T47D (Supplementary Fig. 1) and T47D fulvestrant resistant (Supplementary Fig. 2) when compared with control (untreated), non-senescent cells (Supplementary Fig. 3), BC cells treated with naïve PBMC or their medium (Supplementary Fig. 4, Supplementary Fig. 5, Supplementary Fig. 6) or non-carcinogenic MCF10A breast cells (Fig. 5C-D).

Furthermore, MDA-MB-231-derived primed-PBMC or PBMCcm did not produce death in the MDA-MB-231 BCSen cells (Fig. 5A-B). In contrast, MCF7-derived PBMCcm produced cell death in Sen-MDA-MB-231 indicating that although MDA-MB-231 are not capable to induce a lethal immune response, they remain sensitive to the immune attack.

Also, PBMC that were directly exposed to NS1643 did not induce cell death in BC cells (Supplementary Fig. 7), emphasizing that the SASP-mediated immune response is not mediated by a putative NS1643-dependent activity on PBMC. These findings highlight the specificity of the Kv11.1/SMS-dependent immune response to the molecular characteristics of ER-positive cancer cells and show that this event is mediated by both cellular and chemical factors released by PBMC.

SASP promotes phenotypic change in PBMC

We aimed to understand the specific mechanism linking PBMC-dependent chemical activation of cell death in BC^{Sen}. To this end, a phenotypic characterization of PBMC exposed to SMS was designed. PBMC were treated with SMS-medium derived from MCF7, $MCF7^{D538G}$, T47D, $T47D^{Ful}$, MDA-MB-231 or the non-carcinogenic MCF10A (control) cells that had been treated with NS1643. The CD45RA and CCR7 markers were used to categorize central and effector memory phenotypes in both $CD4^+$ (Fig. 6A-C) and $CD8^+$ (Fig. 6D-E) cells. We observed a significant decrease in the percentage of T naive population

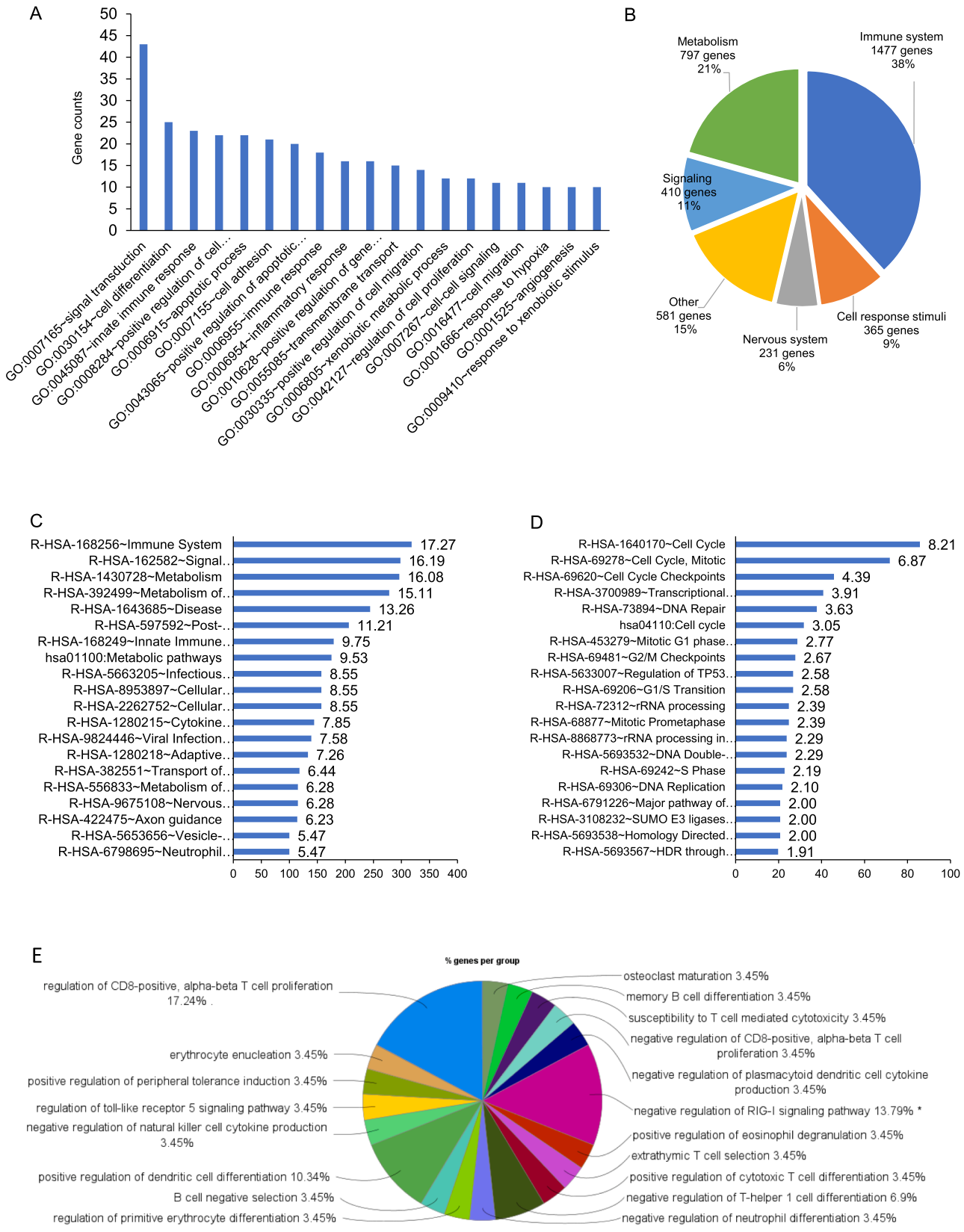


Fig. 1. Activation of Kv11.1 upregulates genes involved in the immune response. A) Gene ontology (GO) enrichment analysis of gene sets from MCF7 BC cells treated with the Kv11.1 potassium channel activator NS1643 (50μM/24Hr). B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (genes expressed in percentage of total). C) REACTOME pathway enrichments of upregulated genes or D) downregulated genes in MCF7 cells treated with NS1643. E) Interrelation analysis of upregulated genes.

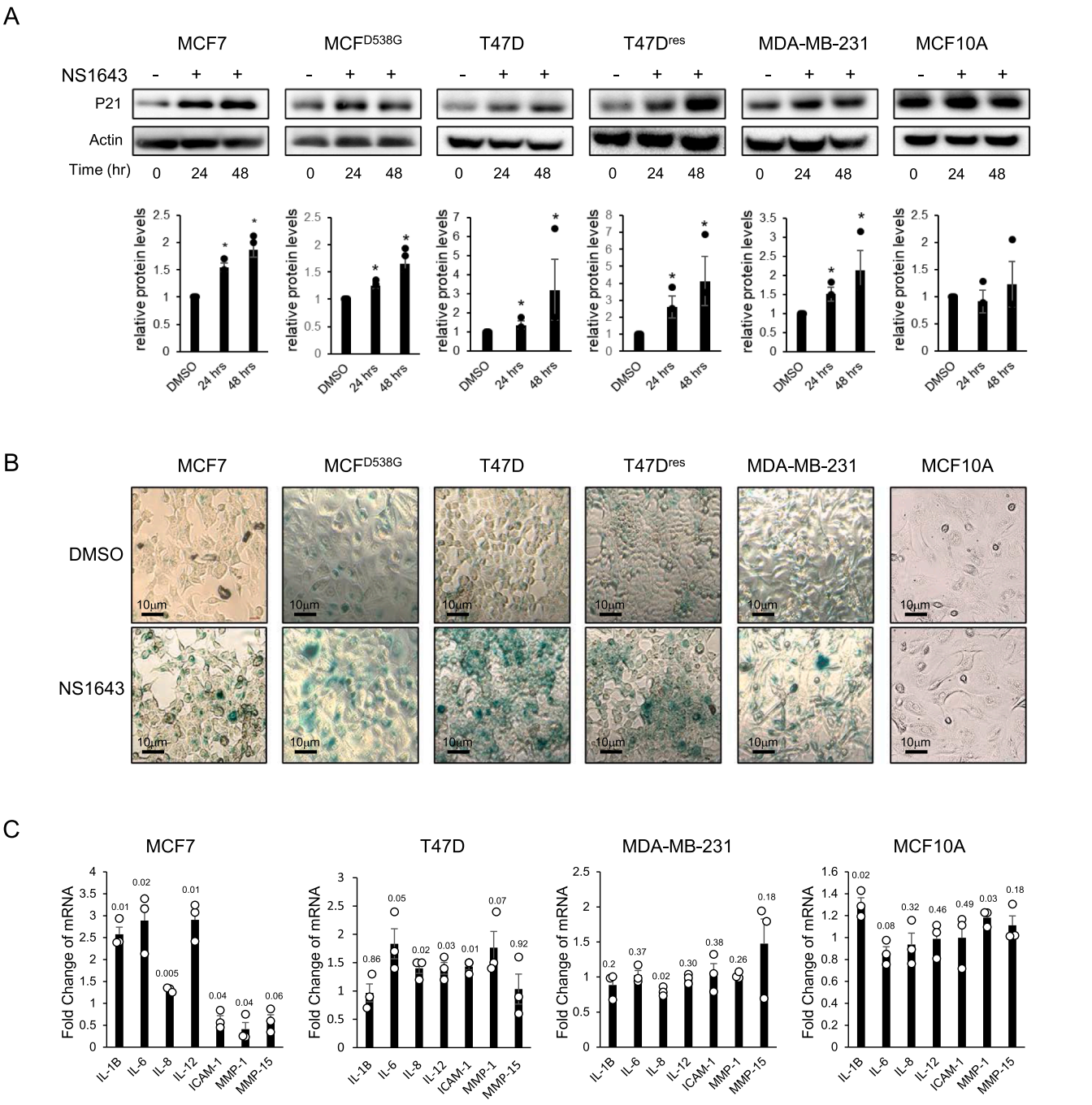


Fig. 2. Effects of NS1643 on senescent markers. A) Western blot showing the effect of NS1643 (50μM) treatment at the indicated times on the expression of the senescent marker p21 in the ER-positive MCF7, MCF7 endocrine resistant, T47D, T47D endocrine resistant, non-carcinogenic MCF10A and in the ER-negative MDA-MB-231. Graph bars indicated quantification of the events in A); B) Representative images of senescence-associated β-galactosidase (SA-β-gal) staining of different BC cells type, and non-carcinogenic breast cells treated with NS1643 (50μM/24hr). C) Effect of NS6143 (50μM/24hr) on senescent associated markers in ERpos MCF7, T47D cell lines; ERneg MDA-MB-231 or MCF10A cell lines by RT-PCR.

(CD45RA+/CCR7+) and a significant increase in the percentage of T cell effector memory (TEM; CD45RA-/CCR7-) in both CD4+ and CD8+ subpopulations when compared to the control in PBMC treated with SMS from ER-positive BC^{Sen} (Fig. 6A-E; Supplementary Fig. 8) independent of their SERM/SERD resistance (Fig. 7A-C ($MCF7^{D538G}$). Interestingly, a more pronounced change was observed in the CD4+ phenotype when compared with CD8+. In contrast, no change was observed in PBMC phenotype after exposure to SMS from MDA-MB-231 (Supplementary Fig. 9) or MCF10A (Supplementary Fig. 10). Furthermore, to understand weather NS1643-dependent PBMC phenotypic change is specific to the Kv11.1 channel activation, we induced a senescent phenotype in MCF7 cells by using the CDK4/6 inhibitor palbociclib (Supplementary Fig. 11A & B). Interestingly, the effect of palbociclib on SASP was significantly smaller when compared with NS1643. Furthermore, when we exposed PBMC to the medium from these cells, we did not observe any significant phenotypic changes in either CD4+ or CD8+ cells. We conclude that Kv11.1 activation in ER-positive BC cells produced a specific SMS that can induces a phenotypic change in the CD4+ lymphocytes subpopulation.

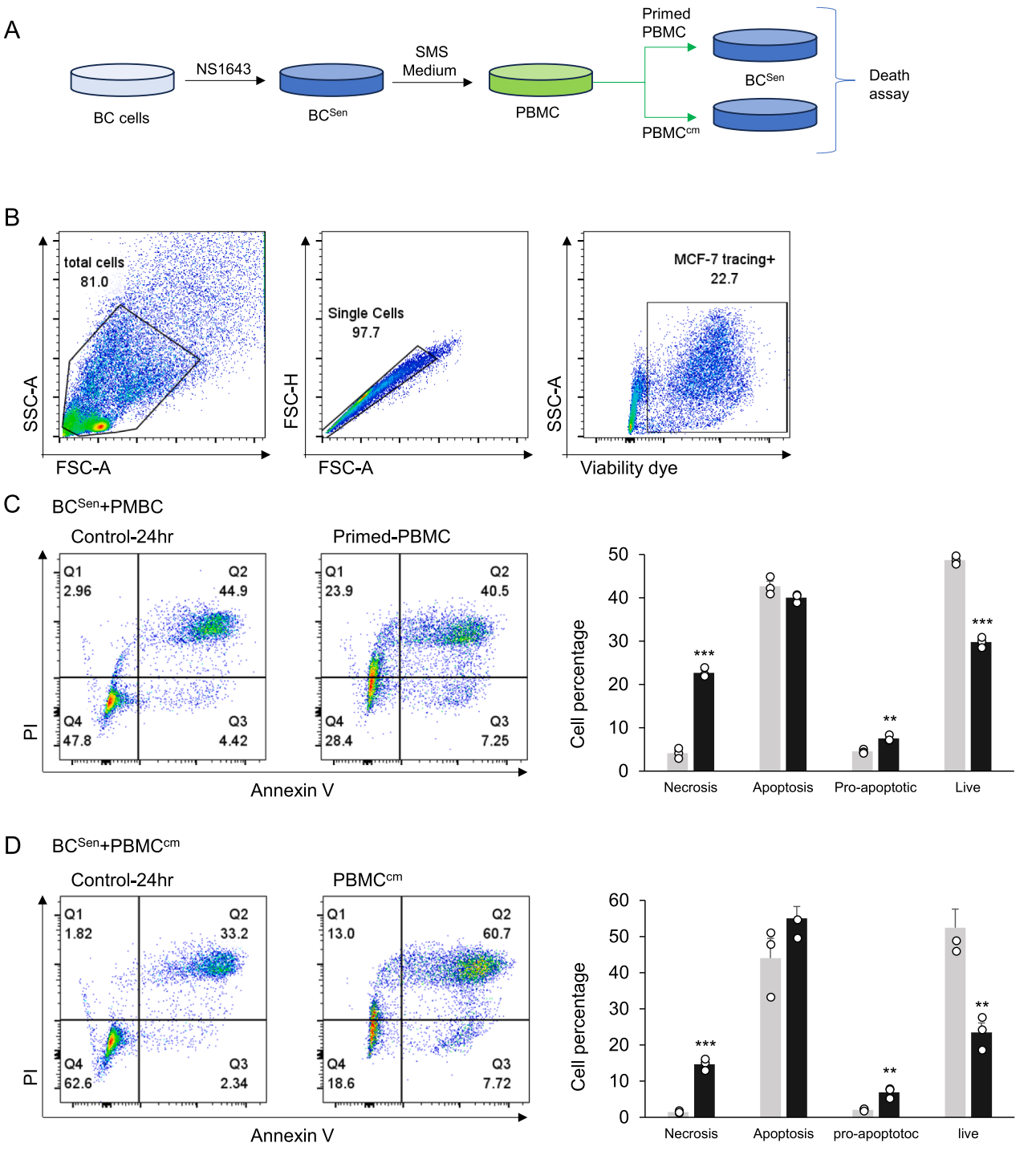


Fig. 3. Senescence-messaging secretome from senescent ER-positive BC cells activates a lethal immune response. A) Schematic representation of the experimental set-up (SMS: senescence-messaging secretome; BC: breast cancer cells; BC^{Sen}: Senescent breast cancer cells after exposure to NS1643; PBMC: peripheral blood mononuclear cells; Primed-PBMC: PBMC treated with BC^{Sen} medium; $PBMC^{cm}$: Medium from PBMC treated with SMS). B) Gating strategy to define the discrimination between effector (PBMC) or target (MCF-7) cells and cell death. B) Flow cytometric analysis of Annexin V/PI-stained in cells treated with primed-PBMC or C) $PBMC^{cm}$. Graphs on the right side indicate quantification of the events in C and D.

Kv11.1/SASP activates Type 1 helper

To explore the clinical relevance of our findings, we investigated the correlation between breast cancer patient prognosis and immune cell infiltration in tumor tissues by using an in silico immune score model (KM-plotter). Data from TCGA database provided the cellular content of CD4+, CD8+, type-1 T-helper cells (Th1), and type-2 T-helper (Th2) cells in BC. We found that the association between KCNH2 and the CD4+ Th1 immune cells had a significant impact on overall survival with high expression of the channel indicating better prognosis (Fig. 8A). These data suggest that Th1 helper cells play a critical role in mediating an immune response against BC.

T helper 1 (Th1) plays a crucial role in suppressing tumor growth through the production of cytokines, such as tumor necrosis factor-alpha (TNFα). Because our results suggest that CD4+ cells might mediate the Kv11.1/SASP immune response against BC^{Sen} we monitored changes in

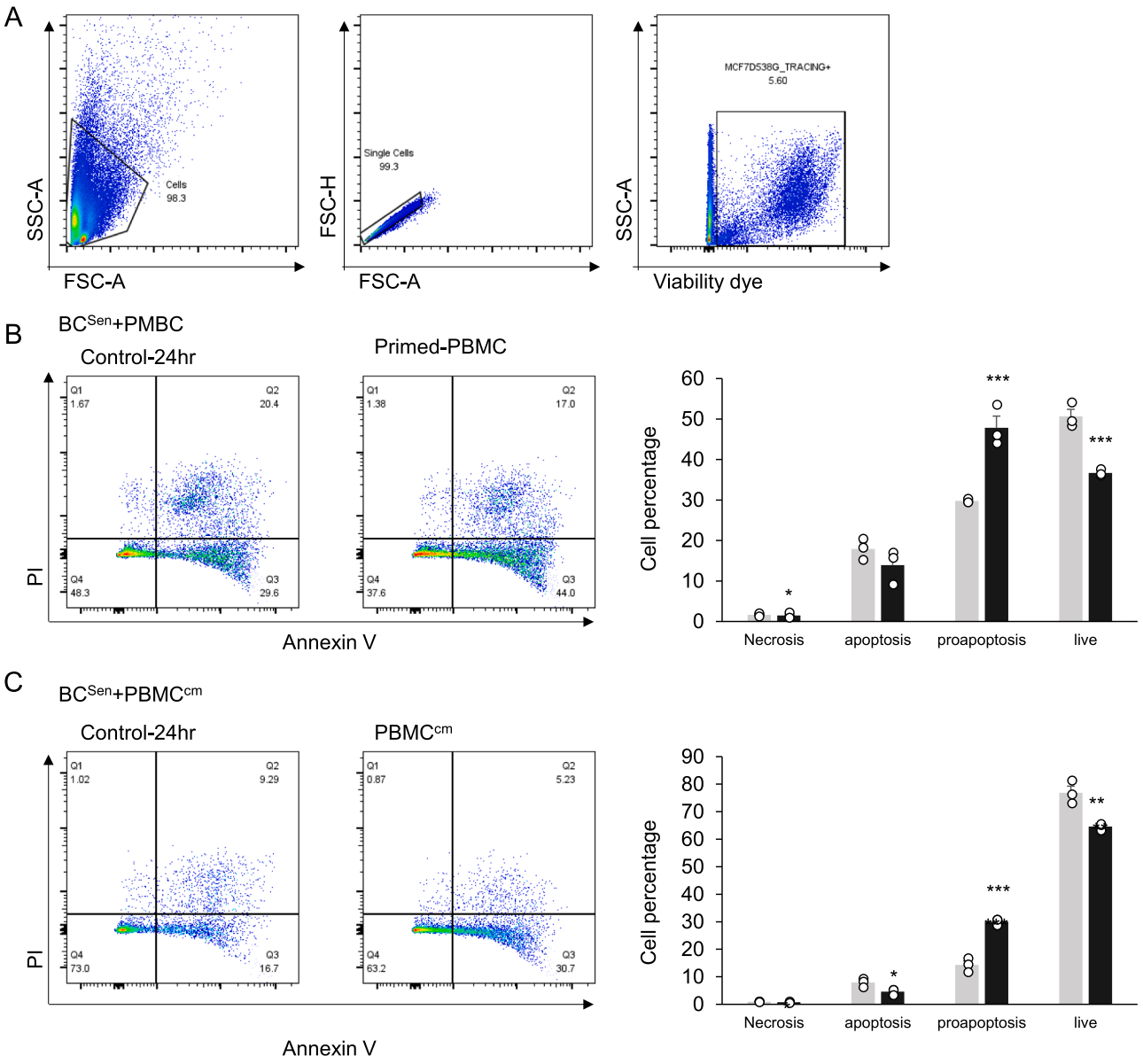


Fig. 4. Senescence-messaging secretome from senescent/SERD resistant ER-positive BC cells activates lethal immune response. A) Gating strategy to define the discrimination between effector (PBMC) or target ($MCF7^{D538G}$) cells and cell death and quantification are shown for cellular coculture assay. B) Flow cytometric analysis of Annexin V/PI-stained in cells treated with primed-PBMC or C) $PBMC^{cm}$. Graph on the right side indicate quantification of the events in B, C.

a Th1 subset of markers, including CD4+/IL12Rβ2 (2), CD4+TIM3+ (3), and CD4+TNFα (4). We found that PBMC treated with Kv11.1/SASP (Fig. 8B-D) showed a significant increase in all markers compared with control, suggesting that SMS medium activates a phenotypic change in the Th1 subpopulation of PBMC.

Kv11.1/SASP-dependent immune response is mediated by TNFα

To delve deeper into the functional role of Th1 in mediating the SASP-dependent immune response, we monitored TNFα production by PBMC. An ELISA assay was performed to assess TNFα concentration in the media where PBMC were treated with SMS from MCF7, $MCF7^{D538G}$ or T47D. We found that with all treatments, PBMC produced a significant increase in TNFα levels when compared to the control (Fig. 9A-B; Supplementary Fig. 11). Conversely, media from plates where BC or PBMC cells were treated with NS1643 did not show a significant change in TNFα compared to the control. These data indicate that Kv11.1-SASP induces PBMC to produce TNFα.

Because treatment of BC^{Sen} with $PBMC^{cm}$ resulted in cell death and because TNFα activates a death pathway through the TNFαR1, we evaluated the expression level of TNFα receptor 1 in BC^{Sen}. We found that NS1643 treatment produced a significant increase of TNFαR1 protein level (Fig. 9C). Additionally, treatment of these BC^{Sen} with $PBMC^{cm}$ resulted in activation of the TNFαR1-dependent death pathway as indicated by increased phosphorylation of MLK (Fig. 9D). Remarkably, this event was completely inhibited by the addition of Enbrel. Furthermore, Enbrel completely inhibited the $PBMC^{cm}$ lethal effect as indicated by the lack of changes in the annexin V assay. These findings underscore the critical role of TNFα in promoting the Kv11.1-SMS-dependent lethal immune response (Supplementary Fig. 12).

Discussion

Cellular senescence is a stress-induced phenotype that is recognized as a potent anticancer mechanism that arrests proliferation of malignant cells. The senescent phenotype can be induced by a plethora of chemical

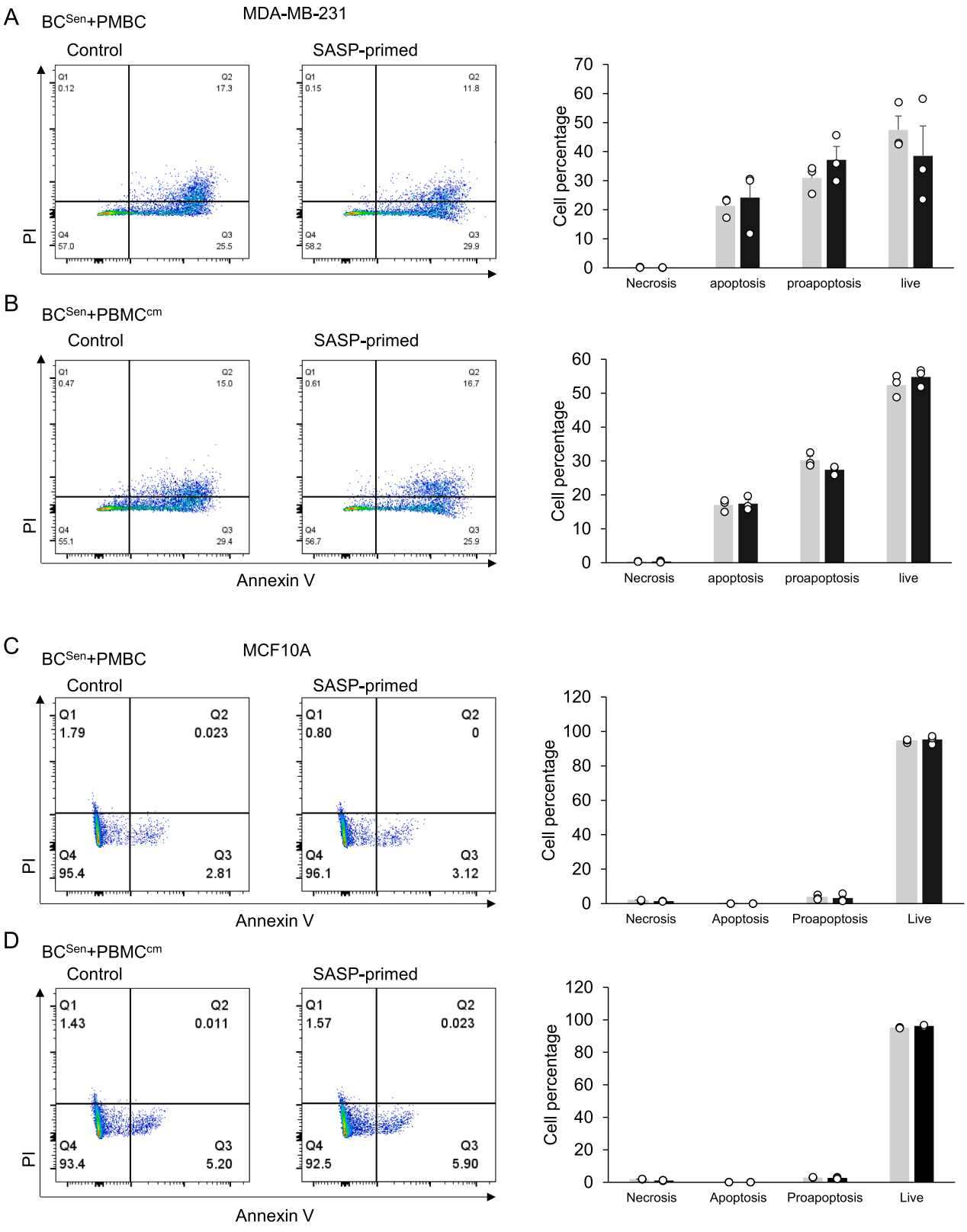


Fig. 5. Senescence-messaging secretome from senescent ER-negative MDA-MB-231 or non-carcinogenic MCF10A fails to activate a lethal immune response. A) Flow cytometric analysis of Annexin V/PI-stained in MDA-MB-231 cells treated with primed-PBMC or B) PBMC^{cm}. C) Flow cytometric analysis of Annexin V/PI-stained in MCF10A cells treated with primed-PBMC or D) PBMC^{cm}. Graph on the right side indicate quantification of the events in A,B,C,D.

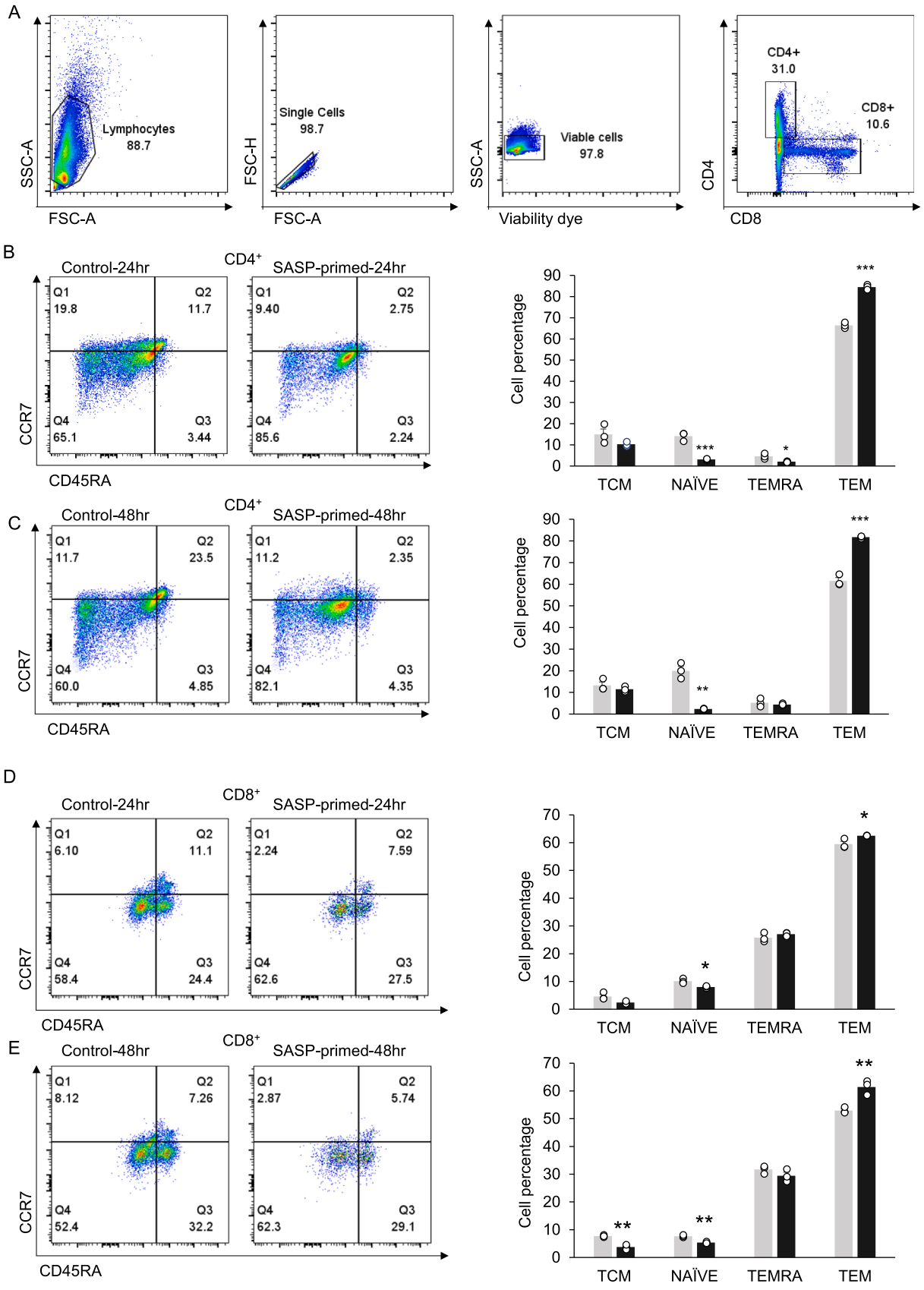


Fig. 6. A) Senescence-messaging secretome from ER-positive BC cells induce a phenotypic change in PBMC. Representative gating strategy. Lymphocytes were gated based on SSC-A vs FSC-A and singlets were selected from the FSC-A versus FSC—H dot plot. CD4+ cells were selected from CD4/CD8 plot. B) Representative Flow cytometry analysis showing a phenotypic change of CD4+ population for 24 hr and C) 48hr or D-E) CD8+ population upon SASP-medium treatment and quantification for 24hr and E) 48 hr. (n = 3; p*<0.05; **<0.001; ***<0.0001).

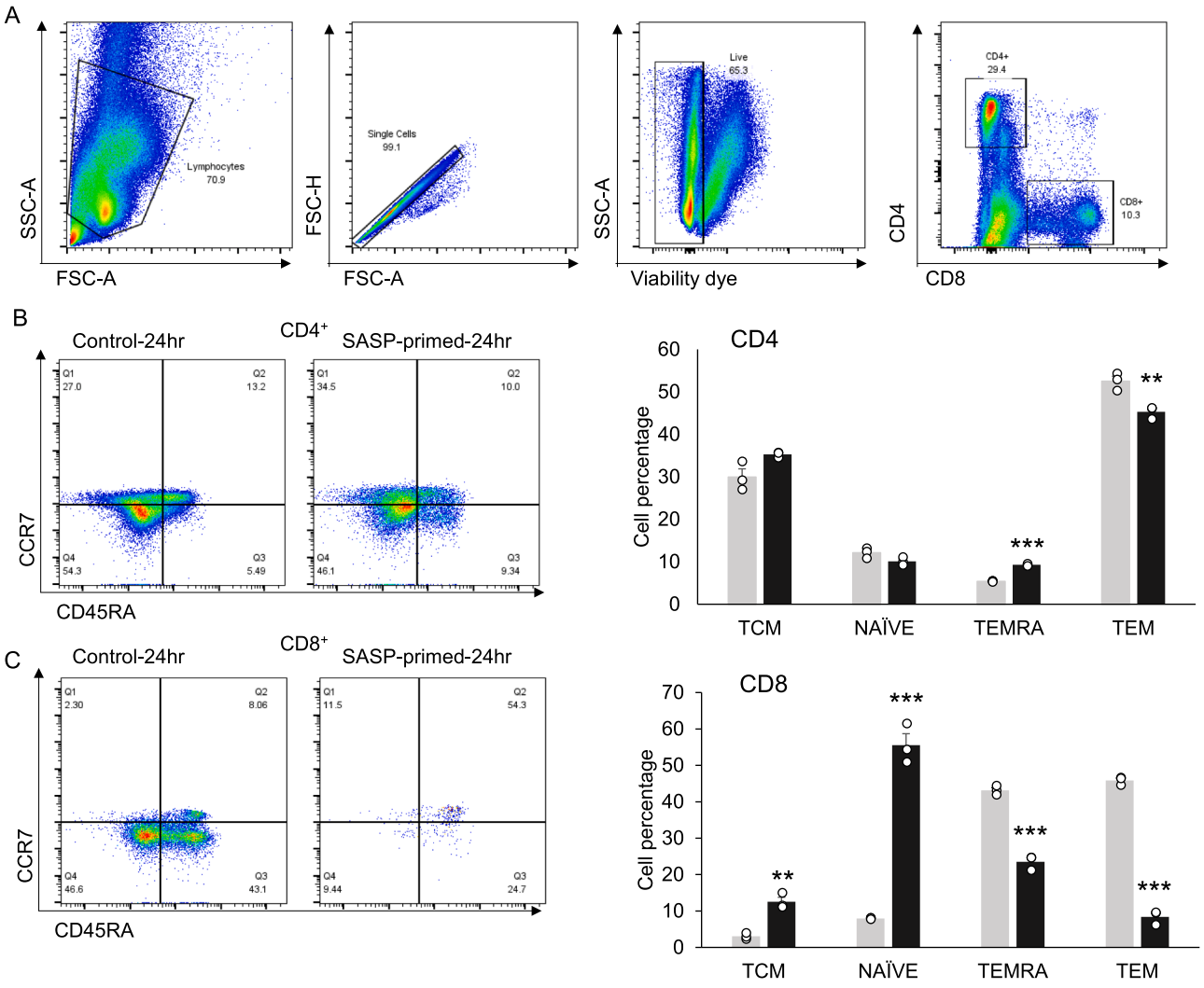


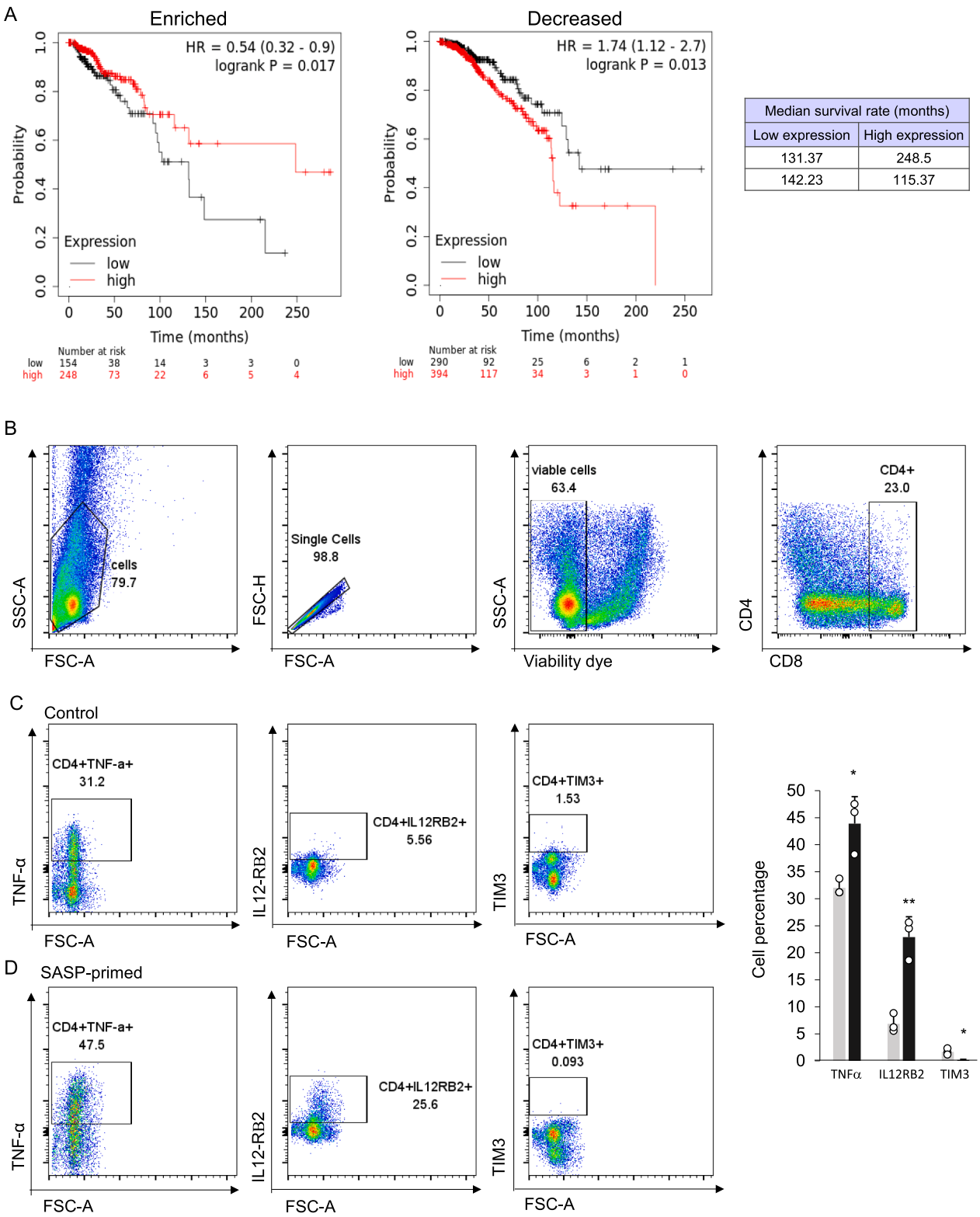
Fig. 7. A) Senescence-messaging secretome from SERD resistant BC cells produce a phenotypic change in PBMC. Representative gating strategy. Lymphocytes were gated based on SSC-A vs FSC-A and singlets were selected from the FSC-A versus FSC—H dot plot. B) CD4+ cells were selected from CD4/CD8 plot. Representative Flow cytometry analysis showing a phenotypic change of CD4+ population or C) CD8+ population upon SASP-medium treatment and quantification for 24hr and C) 48 hr. ($n = 3$; p*<0.05; **<0.001; ***<0.0001).

and physical stressors which can be used for therapeutic intervention. We have previously established that alteration of ionic gradients by pharmacological stimulation of potassium ion channels induces cellular senescence in BC. Therefore, this approach could be considered as a potential therapeutic strategy against BC.

Nevertheless, several studies have shown controversial results on the role of the secretory activities of senescent cells resulting in a heterogenous outcome. For example, SASP can promote or suppress tumor growth by altering immune surveillance. The comprehensive analysis of our experimental results provides valuable insights into the intricate interplay between ion channels activity, cellular senescence, and the immune response in BC cells. Overall, our data indicate that induction of a senescent phenotype in BC, specifically though activation of the Kv11.1 potassium channel, produces a lethal immune response against the senescent cells. Therefore, our data support the hypothesis that the Kv11.1-dependent cellular senescence promotes tumor immune surveillance.

The novelty of our findings lies in the discovery that CD4+ *T* cells can directly kill tumor cells through contact-independent TNFα production, presenting a new therapeutic avenue that could overcome current treatment limitations. While we cannot entirely exclude the involvement of various PBMC subpopulations in this lethal immune response, our data highlight the predominant role of Th1 cells. It is common practice to add IL12 in the PBMC media to enhance immune response. In our experiments we have deliberately avoided this practice. Therefore, Th1-dependent response could be attributed to the significant increase of IL12 in the NS1643-dependent SASP from ERpos BC cells. However, the specific role of IL12 in this process is out of the scope of our work and more detailed experiments need to be performed to better understand this process. Specifically, we elucidated a relation between a K^+ channel dependent activation in cancer cells and immune response driven by TNFα, which adds a crucial layer to our understanding. The increased production of TNFα by PBMCs, alongside the upregulation of TNFα receptor 1 in senescent breast cancer (BC) cells, establishes a direct link between Kv11.1 activation, SASP, and the Th1/TNFα-mediated immune response (Supplementary Fig. 12).

Remarkably, we found that pharmacological stimulation of the Kv11.1 potassium channel induced a senescent phenotype in BC cells, independent of molecular features like ER and p53 status or resistance to standard-of-care therapies such as SERMs or SERDs. However, the Kv11.1-dependent SASP from ER-negative BC cells failed to promote immune polarization. Nevertheless, our experiments demonstrated that when PBMCs were exposed to SASP from ER-positive BC cells, they polarized and exhibited lethal effects on ER-negative senescent BC cells



Median survival rate (months)	
Low expression	High expression
131.37	248.5
142.23	115.37

Fig. 8. Kv11.1-dependent SMS produces Th1 polarization. A) Kaplan–Meier plots showing overall survival in ER-positive BC patients with enriched or decreased T helper and high (red) or low (black) expression of KCNH2 (Kv11.1). Table in the right side indicates median survival rate in the low expression or high expression cohorts. B) Representative gating strategy. Lymphocytes were gated based on SSC-A vs FSC-A and singlets were selected from the FSC-A versus FSC—H dot plot. CD4+ cells were selected from live cells plot. CD4+TNF-α +, CD4+IL12-RB2+ and CD4+TIM3+ are shown in control (C) and SASP-primed (D) PBMC cells. Representative Flow cytometry analysis showing a phenotypic change of CD4+Th1 population upon SASP-medium treatment and quantification for 24hr ($n = 3$; p*<0.05; **<0.001; ***<0.0001).

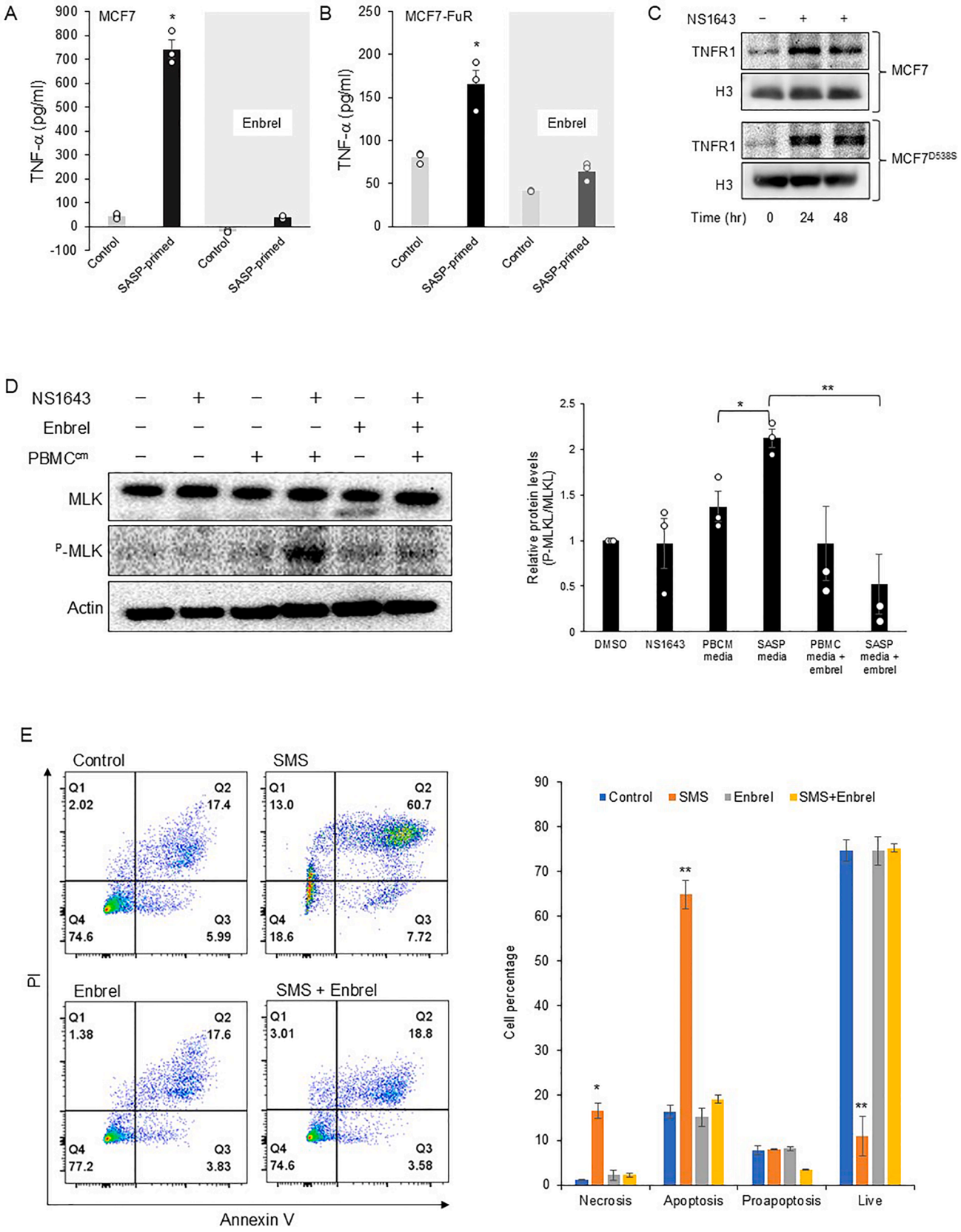


Fig. 9. Kv11.1-dependent immune response is mediated by TNFa. A) Histogram shows TNF-alpha levels in the medium obtained from PBMC treated with SMS medium from MCF7 or B) MCF7^{D538G} alone or with the TNFa blocker Enbrel ($n = 3$; p*<0.05; **<0.001; ***<0.0001). C) Western blot showing the effect of NS1643 (50μM) on the TNFR1 after 24 or 48hr treatment. D) Western blot showing the effect of NS1643 or Enbrel alone or in combination with PBMC^{cm}.

underscoring the specificity of the immune response to ER-positive senescent cells. These results indicate that although Kv11.1-induced senescence in TNBC cells do not produces immune cells polarization, they retain sensitivity to an immune response. Furthermore, inducing senescence by exposure to palbociclib rather than NS1643 did not produce a significant change of the SASP markers when compared to the effects of NS1643 or PMBC polarization. These data suggest that the immune response that we observed via NS1643 is $K+$ gradient and/or Kv11.1 specific. Furthermore, suggests that SASP from ER-positive BC cells contains unique PBMC-polarizing factors, making it more effective at engaging immune surveillance than SASP from ER-negative BC cells. Identifying these key factors will be the focus of future research.

Our study also provides compelling evidence that CD4+ *T* cells produce Th1-specific tumoricidal cytokines in response to senescence induced in ER-positive BC cells through Kv11.1 activation. This mechanism presents an opportunity to explore the therapeutic potential of TNFα administration against ER-positive BC, though systemic TNFα toxicity remains a major hurdle. Significant efforts are required to mitigate these toxic effects to make TNFα a viable therapeutic option.

In conclusion, our findings reveal the intricate interplay between Kv11.1 channel activation, SASP induction, and the resulting immune response in ER-positive breast cancer. This work not only advances our understanding of the molecular mechanisms involved but also opens the door for exploring Kv11.1 modulation as a strategy for immunotherapy in breast cancer. Further studies are needed to fully unlock the therapeutic potential of targeting Kv11.1 in ER-positive BC and its impact on the immune microenvironment.

Materials and methods

Human PBMC

Peripheral blood mononuclear cells (PBMCs) were purchased from Human Peripheral Blood Leukopak (STEMCELL™). The whole blood was mixed in a 1:1 volume ratio with PBS+10% FBS at room temperature. Isolated PBMCs were obtained according to standard protocol. In short, the diluted blood was gently layered on top of the density gradient medium, ensuring that the two layers were not mixed. PBMCs were carefully collected from the Ficoll-Paque-plasma interface by inserting the pipette directly through the upper plasma layer to the mononuclear cells at the interface into a 50 mL tube and then washed twice (500 × *g*, 10 min, 4°C) with RPMI1640 medium (Thermo fisher Scientific) containing 10% FBS (RPMI-FBS) before being resuspended in RPMI-FBS supplemented with 1% penicillin/streptomycin. PBMCs were counted and their viability was assessed using trypan blue and a hemocytometer. Freshly isolated PBMCs were cryopreserved in a solution of 90% FBS and 10% DMSO in liquid nitrogen for subsequent testing [21,22]. PBMC cells were isolated from blood of two distinct leukopaks and 3 healthy donors. All donors were healthy and without any history of cancer or cancer treatment. No additional IL12 was added during experimentation.

Cell lines

Human breast cancer cell lines MCF-7 and T47D were purchased from ATCC while T47D FULR and MCF7 FULR cell lines were a generous gift from Dr. Shahin Ozgur. Cells were maintained at 37°C and 5% CO2 in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% fetal bovine serum, 1% Penicillin (100 mg/ml) and streptomycin (100 mg/mL) antibiotics.

Flow cytometry staining and acquisition

Following purification, 100 µL of each fraction of original samples along with positive controls were stained. This involved adding 100 µL of FACS buffer, 5 µL of each human antibody from 5-Color central/effector memory Immunophenotyping cocktail, which includes CD4 conjugated to Ef450(clone OKT4, eBioscience™), CD8 conjugated to APC (clone SK1, Biolegend), CD45RA conjugated to FITC (clone HI100, bdBioscience), CD197(CCR7) conjugated to PE (clone G043H7, Biolegend) and Viability dye conjugated to Ef506 (1:1000, eBioscience™). For Th1 characterization we used CD4 conjugated to Ef450 (clone OKT4, eBioscience™), anti-hu CD366(TIM3) conjugated to FITC (clone F38-2E2, eBioscience™), anti-h/m IL12RB2 conjugated to PE (R&D systems) and anti-hu TNF-α conjugated to APC (clone Mab11, eBioscience™). Unstimulated frozen PBMCs were retrieved from liquid nitrogen, thawed, and resuspended in supplemented RPMI. The PBMCs were then counted and primed with 48 h-SASP/control primed media for 24h. Subsequently, 1 million SASP/control primed PBMC cells were washed with PBS and utilized for central/effector memory or Th1 immunophenotyping. After washing, cells were incubated with 50 µl FcR blocking reagent for 20 min (1:625 in FACS buffer, Miltenyi Biotec) on ice in the dark. Next, the cells were washed twice with 200 µl of FACS buffer and stained with the central/effector memory surface antibody panel: CD4, CD8, CD45RA, or the Th1 surface antibody panel: CD4, anti-hu CD366(TIM3), anti-h/m IL12RB2. The viability dye was used with surface antibodies in central/effector memory panel staining.

After a 30-minute incubation on ice in the dark, cells were washed twice with FACS buffer and fixed with 200 µl of Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (1:3) for 30 min. The cells were then resuspended and washed two times with Permeabilization Buffer (10X) (eBioscience™). Next, they were stained with the central/effector memory intracellular antibody panel: CD197 (CCR7) or the Th1 intracellular antibody panel: anti-hu TNF-α and incubated for 30 min on ice in the dark. Finally, stained cells were transferred to 5 ml round-bottom polystyrene tubes (Corning Inc.) and stored on ice while resuspending them in PBS before acquisition on an LSR Fortessa X-20 cell analyzer using FACS Diva software (BD Biosciences, San Jose, CA, USA) (3).

One million cells of 24h-SASP/control primed PBMC cells/media were cocultured with 72h NS1643 treated/untreated MCF-7 and MCF7FULR for the Effector (E): target (T) ratio of 10:1 and T47D, T47DFULR for the E:T of 6:1. Twenty-four hours later, whole cells were stained with Cell TraceTM Violet cell proliferation kit, Annexin V conjugated to FITC and PI, incubated at room temperature for 20 min, and signals were acquired using an LSR Fortessa X-20 cell analyzer with FACS Diva software (BD Biosciences, San Jose, CA, USA). The data were analyzed with FlowJo Version 10.2 software and plotted using Excel software.

Flow cytometry gating strategy

Gating hierarchy was applied into both PBMCs and cancer cells. An illustrative gating strategy for central/effector immunophenotyping is presented in Fig.s 2-5, outlining consecutive steps for isolating the CD4+ population, culminating in the identification of naïve, central and effector memory T cell subsets [23]. Second gating strategy was used to define the discrimination between effector (PBMC) or target (cancer) cells and cell death. Total cells including effector and target cells were gated based on SSC-A versus FSC-A and Cell TraceTM Violet cell proliferation kit (Thermo fisher scientific) was used to discriminate target population from PBMC. Propidium iodide versus Annexin V staining was applied to selected target population in cellular and Media assay (Fig. 7-9). All experiments are conducted in triplicate [24]. Third gating strategy was used to characterize Th1. Lymphocytes were gated based on SSC-A vs FSC-A and singlets were selected from the FSC-A versus FSC—H dot plot. CD4+ cells were selected from live cells plot and CD4+TNF-α +, CD4+IL12-RB2+ and CD4+TIM3+ vs FSC-A are shown separately in control and SASP-primed PBMC cells (Fig. 5 and 6) [25–27].

Immunophenotyping and killing assay

We initiated the experiments by seeding 1 million cancer cells in 10 cm dishes, aiming for a confluency of 60%. The cells were then treated with NS1643 (50 μM) every 24 h for 48 h. Then, we collected the supernatant media from NS1643-treated cancer cells (SASP) and non-treated cancer cells (control). Subsequently, we conditioned the PBMC with this media in 6 wells plate after a centrifugation step (2000 rpm for 2 min). Notably, we refrained from using external factors such as anti-CD3/CD28 or IL-2 to activate the PBMC cells before coculturing. The treatment of cancer cells with NS1643 was continued for an additional 24 h. We utilized 24-hour SASP/Control-primed PBMC for central/effector memory and Th1 immunophenotyping.1 million of PBMC cells were then cocultured with 72-hour NS1643-treated/untreated cancer cells. Subsequently, we stained the cells after 24 h with PI and Annexin V for the killing assay or conducted an ELISA to detect TNF-α. For intracellular staining, we analyzed PBMCs without stimulation using PMA or ionomycin. It's important to highlight that in the traditional approach, researchers commonly employed PMA, ionomycin, and brefeldin A for this particular purpose but due to the drastic change in ionic current in cells induced by using PMA or ionomycin, we opted to avoid doing so [28].

Enzyme-linked immunosorbent assay (ELISA)

The supernatant from PBMCs cocultured with breast cancer cells was utilized for TNF-α measurements using an ELISA kit (Human TNF-alpha DuoSet ELISA, R&D Systems) following the manufacturer's instructions. In brief, 100 μl of the cell supernatant was applied to 96-well plates pre-coated with TNF-α capture antibody for overnight. After washing, bound proteins were detected by adding human TNF-α detection antibodies followed by HRP-conjugated streptavidin. Subsequently, a substrate solution, defined as a 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine), was added, and the Optical Density was read at 450 nm immediately after adding the stop solution. The lower limit of TNF-α detection was 15.6 pg/mL [29].

GO and pathway enrichment analysis

DAVID (https://david-d.ncifcrf.gov/) was used to analyze candidate DEG functions and KEGG pathway enrichment. GO term enrichment analysis includes biological process (BP), cellular component (CC), and molecular function (MF). Pathway analysis was also carried out using another online database, REACTOME (available online: http://www.reactome.org). Interrelation analysis between pathways was developed using the ClueGo plug-in Cytoscape software. A p value $<.05$ was considered the cut-off criterion.

Statistical analysis

Student t-tests were employed for mean comparisons. Grouped data is presented as mean ± standard error of the mean (SEM) of 3 replicates. The data analysis was conducted by Excel software. Statistical significance was indicated as ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .0001$, or ns for $P > .05$).

CRediT authorship contribution statement

Maedeh Vakili Saatloo: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Davide Delisi:** Validation, Investigation, Formal analysis, Data curation. **Najmeh Eskandari:** Validation, Data curation. **Carsten Krieg:** Conceptualization. **Saverio Gentile:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2025.101148.

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