



Ly-6A-Induced Growth Inhibition and Cell Death in a Transformed CD4⁺ T Cell Line: Role of Tumor Necrosis Factor- α

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Abstract

Ly-6A, a member of the Ly-6/uPAR supergene family of proteins, is a cell adhesion and cell signaling protein. Signaling through Ly-6A activates the cell-intrinsic apoptotic cell death pathway in CD4⁺ T cell lines, as indicated by the release of cytochrome C, and activation of caspases 9 and 3. In addition, Ly-6A induces cytokine production and growth inhibition. The mechanism underlying the distinct cellular responses that are triggered by engaging Ly-6A protein has remained unknown. To examine the relatedness of these distinct responses, we have quantified the production of pro-apoptotic, growth inhibitory and tumor suppressive cytokines, such as TNF- α , TGF- β and a related protein GDF-10, in response to Ly-6A signaling. Anti-Ly-6A monoclonal antibody-induced activation of YH16.33 CD4⁺ T cell line generated low levels of TGF- β and GDF-10 but elevated levels of TNF- α . Blocking the biological activity of TNF- α resulted in reduced Ly-6A-induced apoptosis in T cells. The Ly-6A-induced response in the T cell line was distinct, as signaling through the antigen receptor complex did not cause growth inhibition and apoptosis despite high levels of TGF- β and GDF-10 that were detected in these cultures. Additionally, in response to antigen receptor complex signaling, lower amount of TNF- α was detected. These results indicate the contribution of TNF- α in the observed Ly-6A-induced growth inhibition and apoptosis and provide a mechanistic explanation for the biologically distinct responses observed in CD4⁺ T cells after engaging Ly-6A protein. Additionally, the findings reported here will aid in the understanding of inhibitory signaling initiated by Ly-6A protein, especially in the context of its potential immune checkpoint inhibitory role in T cells.

Keywords Ly-6A · Ly6 · Apoptosis · T cell signaling · TNF- α · TGF- β · GDF-10 · p27kip

Abbreviations

Ly-6	Lymphocyte antigen-6
CD	Cluster differentiation antigen
TCR	T cell receptor
TNF	Tumor necrosis factor
TGF	Transforming growth factor
GDF	Growth differentiation factor
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)
FL1-4	Fluorescence channel 1–4
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

ELISA	Enzyme-linked immunosorbent assay
ANOVA	Analyses of variance

Introduction

Ly-6A, a member of the Ly-6/uPAR supergene family, is reported to exhibit cell adhesion and cell signaling properties (Bamezai and Rock 1995; Brakenhoff et al. 1995; Malek et al. 1986; Rock et al. 1986; Yeh et al. 1987). Based on the reported *in vitro* experiments, Ly-6A overexpression in T cells results in inhibition of antigen-specific clonal expansion of primary CD4⁺ T cells (Henderson et al. 2002). These findings are consistent with the observed moderate hyperproliferation of Ly-6A^{-/-} CD4⁺ T cells in response to stimulation with anti-TCR/CD3 antibodies (Stanford et al. 1997). Engaging the Ly-6A protein on transformed clonal murine CD4⁺ T cell lines with anti-Ly-6A antibody induces distinct cellular processes as assessed by generation of cytokines (requiring gene transcription), apoptosis

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and growth inhibition (Codias et al. 1990; Henderson et al. 2002; Lang et al. 2017; Merćep et al. 1988; Stanford et al. 1997). The growth inhibited cells show production of the cytokine IL-2 and upregulation of the inhibitory cell cycle protein, p27^{kip} without change in p53 expression (Lang et al. 2017). Additionally, Ly-6A signaling induces death in murine T cell lines through a cell-intrinsic apoptotic mechanism as indicated by translocation of cytochrome C to the cytoplasm and the detection of activated caspases 9 and 3 (Lang et al. 2017). We sought to investigate the underlying mechanism for the functionally distinct responses generated in the murine T cell lines. We considered two possibilities. First, by engaging Ly-6A, multiple distinct signaling pathways are initiated resulting in varied functional responses (cytokine production, apoptosis etc.) through a direct cell-autonomous mechanism. Alternatively, Ly-6A induces apoptosis indirectly, through a non-cell autonomous mechanism, first by secreting growth inhibitory/cell death factors that in turn trigger growth inhibition and apoptosis. We report that Ly-6A-induced growth inhibition and apoptosis partly occur by a non-cell autonomous mechanism mediated by tumor necrosis factor (TNF)- α generated after engaging the Ly-6A protein on the clonal CD4⁺ T cell line.

Materials and Methods

Cell Lines and Antibodies

YH16.33, a CD4⁺ T cell hybridoma cell line, was used in our assays (Yeh et al. 1988). Monoclonal antibodies against CD3 ϵ protein (145-2C11) (Leo et al. 1987) and Ly-6A (8G12) (Bamezai 2004) were used to stimulate YH16.33, a CD4⁺ T cell line, as per previously reported protocols (Lang et al. 2017).

Cell Culture/Growth Inhibitory Assays

YH16.33 were expanded in RPMI 1640-based cell culture media supplemented with 10% fetal bovine serum (Sigma-Aldrich, Inc. St. Louis, MO, USA), 2 mM of non-essential amino acids, 100 IU/mL penicillin and 100 IU/mL streptomycin and 0.25 μ g/mL Amphotericin B and 2 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in the presence or absence of antibodies in a 5% CO₂ incubator at 37 °C with approximately 90–95% humidity for 24 h. YH16.33 were sub-cultured and expanded with fresh media one day prior to the day of the experiment. Experimental cell cultures were set up in a 96-well plate (CytoOne, USA Scientific, Orlando, FL, USA) with 2.5×10^4 YH.16.33 cell/well in the presence or absence of antibodies and/or other treatments in a final volume of 200 μ L of culture media.

Growth Inhibition Assays

MTT reagent (Promega, Madison, WI, USA) containing tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was used to quantify growth inhibition and proliferation in our cultures. The blue soluble formazan product observed in cell cultures was assessed by a spectrophotometer plate reader (CLARIO Star, BMG LabTech, NC, USA) at 490 nm (Berridge et al. 2005). The MTT assay quantifies overall metabolic activity that shows a tight association with T cell expansion and response (Chapman et al. 2020; Pearce 2010). The relative viability in cell cultures was calculated by normalizing the absorbance values obtained in the treatment group to the absorbance of the “No Treatment” group using the following formula:

Relative viability (%)

$$= \frac{\text{Average absorbance of treatment group}}{\text{Average absorbance of no treatment group}} \times 100 (\%).$$

The “No Treatment groups” were assigned a relative viability of 100%, while the TNF- α , transforming growth factor (TGF)- β , and growth differentiation factor (GDF)-10 cytokines in the supernatants from the control and treatment group cell cultures were quantified (see below).

Cytokine Assays

Supernatants (100 μ L) from control and treatment cultures were harvested at 24- and 48-h time points and diluted with 400 μ L of culture media in a 96-deep well storage plate (VWR International, Radnor, PA, USA) and stored frozen at –20 °C for cytokine assay. TNF- α (BD Bioscience, San Jose, CA, USA), TGF- β (Total and Free Active), (BioLegend, San Diego, CA), and GDF-10 (MyBioSource, San Diego, CA, USA) were quantified by enzyme linked immunosorbent assay (ELISA) as per vendor’s instructions. Total TGF- β levels were quantified after acidification, neutralization, and further dilution steps to free TGF- β from its inactivating LAP protein (Khalil 1999) prior to quantification by ELISA.

Antibody Blocking Assays

To assess the role of the TNF- α cytokine in growth inhibition of CD4⁺ T cells induced by the anti-Ly-6A antibody, the biological activity of TNF- α was neutralized with an anti-TNF- α antibody (BioLegend, San Diego, CA, USA; Clone MP6-XT22). Cultures with isotype-matched antibody (BioLegend, San Diego, CA, USA) served as controls for determining the specificity in these blocking assays.

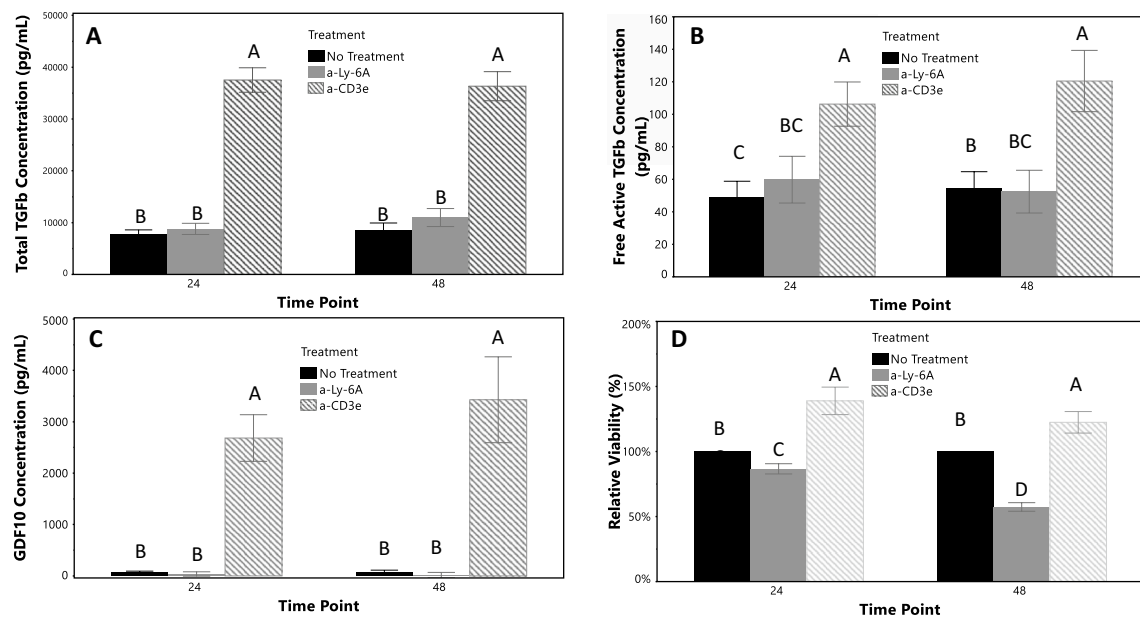


Fig. 1 Production of total TGF- β , free (active) TGF- β and GDF-10 by YH16.33 cells in response to activation through Ly-6A and CD3 ϵ . YH16.33 cells were treated with anti-Ly-6A (gray: a-Ly-6A), anti-CD3 ϵ (white-hatched: a-CD ϵ) monoclonal antibodies, or left untreated (black: No Treatment) (control) for 24 and 48 h, and supernatants were harvested for total TGF- β (A), free TGF- β (B) and GDF-10 (C) quantification by ELISA. The growth inhibitory response of YH16.33 cells in the same assay is shown (D). Data are indicative

of triplicates run during four (active TGF- β) or five (total TGF- β and GDF-10) independent trials. The mean of each treatment group is plotted, with error bars showing standard error. ANOVA analysis was performed with a post hoc Tukey test. Groups with dissimilar connecting letters are significantly different from each other ($p < 0.05$); $n = 4$ or 5. a-Ly-6A anti-Ly-6A antibody, a-CD3 ϵ anti-CD3 ϵ antibody, TGF- β TGF- β

Apoptotic Quantification

Apoptosis in cell cultures was assessed after staining with Annexin V-FITC (BioLegend, San Diego, CA, USA) and Propidium Iodide (PI) (BD Bioscience, San Jose, CA, USA) followed by flow cytometry using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) as previously published (Lang et al. 2017). For each analysis, 10,000 cells were acquired in FL1 (FITC) and FL2 (PE) channels, respectively, without any gating protocols. The absence of staining with Annexin V-FITC and PI enumerates the live cell population. Cells with bound Annexin V-FITC without PI staining mark cells undergoing apoptosis while the plasma membrane is impervious to PI (early apoptosis). The Annexin V-FITC and PI double-positive cells were deemed cells that had undergone apoptosis.

Statistical Analysis

ANOVA analysis with Tukey–Kramer post-test analysis was used to determine the significance in MTT cell proliferation assay and flow cytometric analyses. ANOVA analysis showed differences among group means, while the Tukey–Kramer post-test analysis revealed differences between pairs of means; the null hypothesis was

rejected if the p value was < 0.05 . To determine the significance of cytokine levels from ELISA analysis, two-way ANOVA analyses were performed with a Tukey–Kramer post-test analysis. Connecting letters were reported in conjunction with p values to show significant differences in the cytokine levels among various antibody treatments (treatment groups sharing connecting letters do not show significant difference, whereas treatment groups with different connecting letters are significantly different).

Results

Ly-6A Signaling Promotes Production of TGF- β and GDF-10 Cytokines in a Mouse CD4⁺ T Cell Line

To understand how functionally opposing responses are triggered by Ly-6A signaling in a clonal immortalized CD4⁺ T cell line, we first quantified two growth inhibitory cytokines, TGF- β (as biologically inactive dimer and active monomer) (Fig. 1A, B), and GDF-10, a member of the TGF- β family (Fig. 1C). An average of 8.8 ± 4.2 and 11 ± 6.7 ng/mL total TGF- β was detected at 24- and 48-h time points, respectively. TGF- β produced in response to Ly-6A stimulation was not significantly different from the control

untreated cultures (7.8 ± 3.1 ng/mL at 24 h, $p=0.9989$; and 8.5 ± 5.6 ng/mL, $p=0.9306$ at 48 h). In contrast, anti-CD3 ϵ generated an average of 37.5 ± 9.2 ng/mL and 36.3 ± 10.9 ng/mL of total TGF- β at 24 and 48 h, respectively, a 3.5-fold higher response than the untreated controls. The amount of TGF- β detected in anti-CD3 ϵ treated cultures was significantly higher ($p < 0.0001$) than the untreated controls and anti-Ly-6A treated cultures ($p < 0.0001$) (Fig. 1A). We quantified the free active monomeric form of TGF- β in the same cell cultures. An average of 59 ± 5 pg/mL and 52 ± 5 pg/mL of free active TGF- β was detected in cell cultures treated with anti-Ly-6A antibody at 24- and 48-h time points, respectively. The active TGF- β generated in anti-Ly-6A cultures was not significantly different from the untreated cell cultures over the same time course (Fig. 1B). Anti-CD3 ϵ -treated YH16.33 cells generated 106 ± 47 and 102 ± 65 pg/mL of active TGF- β at 24- and 48-h time points, respectively, which was significantly higher than in the untreated controls (24 h: $p=0.0466$; 48 h: $p=0.0134$; Fig. 1B).

We next sought to assay GDF-10, a TGF- β -like cytokine and another reported ligand for TGF- β RI/II (Ebendal et al. 1998). While Ly-6A expression is known to promote tumorigenesis in epithelial cells by influencing signaling through GDF-10 and TGF- β RI/II receptor signaling axis (Camarata et al. 2015; Cunningham et al. 1995; Upadhyay et al. 2011), its role in T lymphocytes is not. An average of 37 ± 165 and 16 ± 204 pg/mL of GDF-10 was detected in cell cultures treated with anti-Ly-6A at 24- and 48-h post-treatment, respectively (Fig. 1C). GDF-10 detected in anti-Ly-6A treated and untreated control was similar (24-h: 60 ± 1 pg/mL and 48-h: 75 ± 1 pg/mL). In contrast, 45–70-fold higher GDF-10 (24-h: 2684 ± 1757 and 48-h: 3429 ± 3236 pg/mL) was detected in the anti-CD3 ϵ treatment group (Fig. 1C).

Our data show that in response to Ly-6A signaling, YH16.33 cells produce insignificant amounts of GDF-10 and TGF- β , two known growth inhibitory cytokines, and therefore, are less likely to be involved in the observed Ly-6A-mediated growth inhibition (Fig. 1D). This inference is consistent with the observation that significantly higher amounts of GDF-10 and TGF- β are detected in YH16.33 cell cultures in the presence of anti-CD3 ϵ treatment that did not result in growth inhibition (Fig. 1D).

YH16.33 CD4⁺ T Cells Generate TNF- α in Response to Ly-6A Signaling

TNF- α , along with other members of TNF gene family are known to have, depending on the context of stimulation, either growth inhibitory/pro-apoptotic or cell survival effects (Aggarwal et al. 2012; Gough and Myles 2020). The role of TNF- α in mediating the biological effects of Ly-6A signaling is unknown. To examine the role of TNF- α in Ly-6A-induced growth inhibition in YH16.33 cells, we quantified

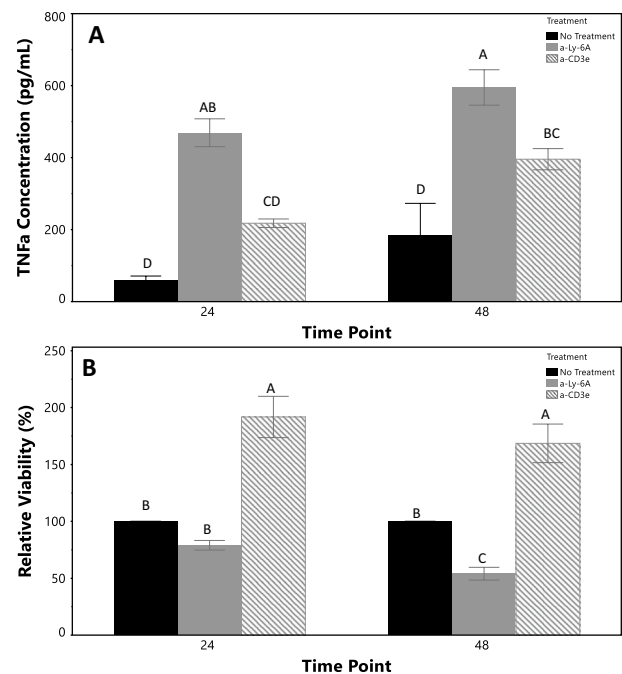


Fig. 2 Engaging Ly-6A induces production of TNF- α in YH16.33 cells. YH16.33 cells were treated with anti-Ly-6A (gray: a-Ly-6A), anti-CD3 ϵ (white-hatched: a-anti-CD3 ϵ), or left untreated (black: No Treatment) (control) for 24 and 48 h, and supernatants were harvested for TNF- α quantification by ELISA (A) and growth inhibition using MTT assay (B). Data are indicative of triplicates run during five independent trials. The mean of each treatment group is plotted, with error bars showing standard error. ANOVA analysis was performed with a post-hoc Tukey test. Groups with dissimilar connecting letters are significantly different from each other ($p < 0.05$); $n=5$. a-Ly-6A anti-Ly-6A, a-CD3 ϵ anti-CD3 ϵ , TNF- α TNF- α

TNF- α production in Ly-6A-dependent growth-inhibited cell cultures (Fig. 2). An average of 469 ± 2 pg/mL and 594 ± 2 pg/mL of TNF- α was detected at 24- and 48-h time points, respectively (Fig. 2A). Significantly ($p < 0.0001$) higher TNF- α was present in anti-Ly-6A antibody stimulated YH16.33 cell cultures than in the untreated control group (24 h: 60 ± 41 pg/mL; 48 h: 184 ± 344 pg/mL). An average of 217 ± 5 pg/mL and 395 ± 1 pg/mL was detected in cell cultures with anti-CD3 ϵ antibody at 24- and 48-h time points, respectively. Anti-CD3 ϵ -induced TNF- α levels were higher than in the untreated cell cultures; however, it was significantly lower than amounts generated in the presence of Ly-6A antibodies (24 h: $p=0.0033$; 48 h: $p=0.0366$; Fig. 2A). Cell cultures used for estimation of cytokines were also examined for cell viability. Figure 2B confirms reduced viability in YH16.33 cell cultures in the presence of anti-Ly-6A antibody relative to the untreated control cultures. Anti-Ly-6A antibody-treated cell cultures show 21% reduced viability when assessed at the 24-h time point, which was not significantly different from the untreated controls ($p=0.381$); however, 48-h anti-Ly-6A antibody treated

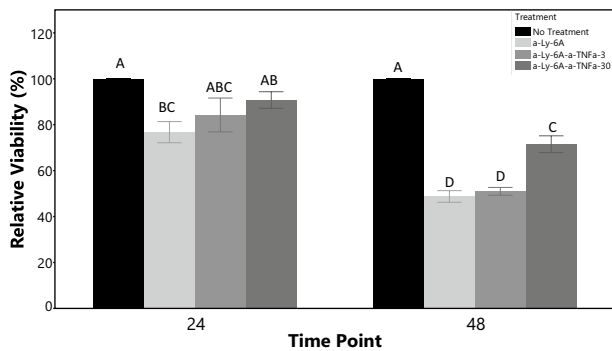


Fig. 3 Effect of neutralizing anti-TNF- α antibody on viability of anti-Ly-6A stimulated YH16.33 cells. Relative viability of YH16.33 in response to treatment with anti-Ly-6A (light-gray: a-Ly-6A), anti-Ly-6A with TNF- α neutralizing antibody at 3 μ g/mL (dark-gray: a-Ly-6A-a-TNF α -3), anti-Ly-6A with TNF- α neutralizing antibody at 30 μ g/mL (gray-black: a-Ly-6A-a-TNF α -30) and untreated cells (black: no treatment) at 24 and 48 h. Cells were treated as specified and proliferation was assessed via MTT assay. The above data are relative to the no treatment control group proliferation and are indicative of triplicates run during four independent trials. The mean of each treatment group is plotted, with error bars showing standard error. ANOVA analysis was performed with a post-hoc Tukey test. Groups with dissimilar connecting letters are significantly different from each other ($p < 0.05$); $n = 4$. No Tx no treatment, a-Ly-6A anti-Ly-6A antibody, a-TNF α anti-TNF- α antibody

cultures showed significantly reduced average viability by 46% ($p = 0.0211$). In contrast, anti-CD3 ϵ antibody treated cultures showed higher cell viability (92% at 24 h and 69% at 48 h) which was significantly higher than in the untreated controls (24 h: $p = 0.0002$; 48 h: $p = 0.0014$).

Neutralizing the Biological Activity of TNF- α Partially Reverses the Ly-6A-Triggered Growth-Inhibition in YH16.33 Cells

We next sought to examine the functional role of TNF- α in anti-Ly-6A antibody-induced growth inhibition and apoptosis by blocking TNF- α activity with a neutralizing anti-TNF- α monoclonal antibody. The relative viability of YH16.33 cells treated with the anti-Ly-6A antibody in the absence of anti-TNF- α antibody was significantly reduced (Fig. 3), as reported before (Lang et al. 2017). Inclusion of anti-TNF- α antibody, at 3 and 30 μ g/mL concentrations, in anti-Ly-6A treated cell cultures resulted in moderately enhanced cell survival when assessed at the 24-h time point. However, this increase was not significant (Fig. 3). At the 48-h time point, a 72% increase in viable cells was observed in anti-Ly-6A treated cell cultures in the presence of 30 μ g/mL anti-TNF- α antibody, when compared to the untreated control group ($p = 0.0049$). At the 48-h time point, moderately higher cell viability was observed at lower concentration of anti-TNF- α (3 μ g/mL), however,

this was not statistically significant when compared to the anti-Ly-6A treated cultures in the absence of anti-TNF- α antibody (Fig. 3). Though treatment with anti-TNF- α neutralizing antibody at 30 μ g/mL increased the average relative viability of cells in anti-Ly-6A stimulated cell cultures at 48 h, the viability did not equal the untreated control group ($p = 0.0003$) (Fig. 3). Taken together, our data indicate that the higher concentration of anti-TNF- α antibody significantly reversed the Ly-6A-induced growth inhibition in YH16.33 cells at the 48-h time point.

Neutralizing Anti-TNF- α Monoclonal Antibody Partially Reverses Ly-6A-Induced Growth Inhibition by Reducing Cell Death

To assess apoptotic cell death in cell cultures, we stained cells with Annexin V-FITC and Propidium Iodide (PI) followed by flow cytometric analyses. About 27% of the total anti-Ly-6A treated cells were viable (AnnV^{neg}, PI^{neg}), this fraction was significantly decreased ($p = 0.0009$) as compared to the untreated control cultures (Fig. 4A). Consistent with this finding is the observation that the remainder 73% (average) of the anti-Ly-6A antibody treated YH16.33 cells showed an apoptotic (Annexin V^{pos} PI^{neg}) or dead (Annexin V^{pos} PI^{pos}) phenotype at the 48-h time point (Fig. 4B). The combined fraction of dead cells, including the apoptotic (Annexin V^{pos} PI^{neg}) cells in anti-Ly-6A antibody-treated cultures was significantly higher than the untreated control. Adding neutralizing anti-TNF- α antibody, at 3 μ g/mL and 30 μ g/mL, to these cultures increased the proportion of viable cells to 41% and 43%, respectively (Fig. 4A). However, this increase was not significant when compared to the anti-Ly-6A-treated cultures alone or in combination with isotype control antibody (Fig. 4A). An average of 58% and 57% cells in anti-Ly-6A antibody treated YH16.33 cell cultures showed an apoptotic or dead phenotype in the presence of 3 μ g/mL and 30 μ g/mL of anti-TNF- α antibody, respectively (Fig. 4B). This moderately reduced death in the cultures was not significant ($p = 0.6540$) when compared to anti-Ly-6A cell cultures alone or in combination with the isotype control where an average of 72% and 73% of cells were apoptotic or dead (Fig. 4B). Though there was a slight decrease in total apoptosis compared to anti-Ly-6A alone, treatment with anti-TNF- α at both 3 μ g/mL (average 58%) and 30 μ g/mL (average 57%) concentrations did not decrease the populations of cells in apoptosis comparable to cells in the “No Treatment” controls (approx. 72%).

An average of 34–35% of cells undergoing apoptosis (AnnexinV^{pos}PI^{neg}) were detected in cell cultures with anti-Ly-6A antibody, either alone or in combination with the isotype control antibodies at 3 μ g/mL and 30 μ g/mL concentration. This was significantly higher than what was

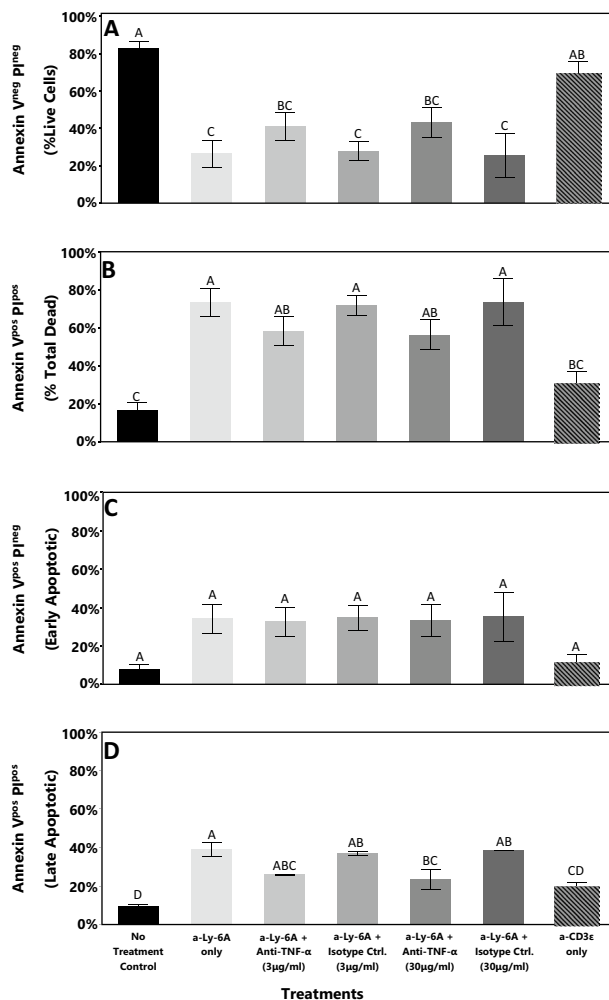


Fig. 4 Effect of neutralizing anti-TNF- α antibody on apoptotic cell death in YH16.33 in response to anti-Ly-6A stimulation. YH16.33 cells incubated with anti-Ly-6A, anti-CD3 ϵ , and anti-TNF- α or isotype control antibody or left untreated for 48 h. Viable and apoptotic cells were analyzed after staining with Annexin V – FITC and Propidium Iodide followed by flow cytometric analysis. The figure represents data from three independent trials, with at least 5000 events per sample. The plotted mean of each treatment group shows percent viable (Annexin-V^{neg}PI^{neg}), early apoptotic (Annexin V^{pos}PI^{neg}) or late apoptotic/dead (Annexin V^{pos}PI^{pos}), with error bars showing standard error. ANOVA analysis was performed with a post hoc Tukey test. Groups with dissimilar connecting letters are significantly different from each other ($p < 0.05$); $n = 3$. *No Treatment* no antibody, *a-Ly-6A* anti-Ly-6A antibody, *a-CD3 ϵ* anti-CD3 ϵ antibody, *a-TNF α* anti-TNF- α antibody, *Ctrl.* control antibody, *PI* propidium iodide

observed in control untreated cell cultures (8%) and cells treated with anti-CD3 ϵ (11%). Inclusion of neutralizing anti-TNF- α antibodies at 3 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ (average 33%) did not alter the proportion of AnnexinV^{pos}PI^{neg} cells in anti-Ly-6A stimulated cell cultures (average 33% at both anti-TNF- α concentrations) when compared to the anti-Ly-6A stimulated cell cultures with isotype control

antibodies (average 35%) ($p > 0.05$) (Fig. 4C). An average of 39% AnnexinV^{pos}PI^{pos} cells were detected in cultures with anti-Ly-6A alone (Fig. 4D). A similar percentage, 37% and 38%, of AnnexinV^{pos}PI^{pos} cells was detected in anti-Ly-6A activated cultures with 3 $\mu\text{g/mL}$ or 30 $\mu\text{g/mL}$ isotype control antibodies, respectively. Isotype control antibody in cell cultures did not significantly alter anti-Ly-6A induced cell death. In contrast, inclusion of neutralizing anti-TNF- α at 3 $\mu\text{g/mL}$ or 30 $\mu\text{g/mL}$ reduced the percentage of dead cells to 26% and 23%, respectively. This reduction was significant ($p = 0.0223$) compared to the anti-Ly-6A treatment group (Fig. 4D). The neutralizing TNF- α reduces the appearance of later apoptotic cells; however, the effects are not complete. Taken together, our data show that engaging Ly-6A on CD4⁺ T cell line promotes secretion of TNF- α , which in turn promotes apoptosis. These data suggest that Ly-6A triggered apoptotic cell death, in part, is triggered by an indirect, non-cell autonomous mechanism. In contrast, anti-CD3 ϵ did not cause significant changes in the viability of YH16.33 cells (Fig. 4A–D).

Discussion

Engaging Ly-6A, expressed on YH16.33 and other CD4⁺ T cell lines, with an activating anti-Ly-6A antibody results in varied cellular responses ranging from growth inhibition, apoptosis and cytokine (IL-2 and IFN- γ) production (Lang et al. 2017). Ly-6A signaling upregulates cyclin-dependent kinase inhibitor p27^{kip1} without altering expression of p53 (Lang et al. 2017). Additionally, Ly-6A activates an intrinsic apoptotic cell death pathway by destabilizing mitochondria, as evidenced by the release of cytochrome C in the cytoplasm and activation of caspases 9 and 3 (Lang et al. 2017). The phenomenon of Ly-6A-triggered growth inhibition, apoptosis, and cytokine release by a clonal T cell line is paradoxical. In this study, we examined the relatedness of the three apparently distinct and conflicting functional responses. We hypothesized the role of a non-cell autonomous mechanism in Ly-6A-induced growth inhibition and apoptosis. We report that the YH16.33 CD4⁺ T cell line secretes TNF- α in response to Ly-6A stimulation, which in turn contributes to the observed T cell growth inhibition and apoptotic cell death in the clonal T cell line. Additionally, TGF- β and GDF-10, members of the TGF family, are minimally detected in these cell cultures and therefore are less likely to contribute to the observed growth inhibitory and apoptotic functional responses.

CD4⁺ T cell lines, when stimulated through Ly-6A, are known to generate cytokines (Bamezai 2004), including IFN- γ (Dumont and Boltz 1987; Snapper et al. 1991).

However, TNF- α production by clonal T cell lines after engaging Ly-6A proteins, and the subsequent biological effects, to our knowledge, are unknown. Our report adds TNF- α to a list of cytokines produced in response to Ly-6A activation. Importantly, anti-TNF- α antibody blocking data (Fig. 3) shows the contribution of TNF- α in Ly-6A-induced apoptosis and growth inhibition of CD4⁺ T cell line by a non-cell autonomous mechanism. These findings are consistent with reports of TNF- α as a growth inhibitory and death factor (Aggarwal et al. 2012) and in its activity in promoting p27^{kip1} expression and caspase 3 activity (Jia et al. 2011). Cellular effects of TNF- α on T cells, as described above, mirror Ly-6A-induced upregulation of p27^{kip}, release of cytochrome C in the cytoplasm, and activation of caspases 9 and 3 (Lang et al. 2017). In contrast, anti-CD3 ϵ antibody stimulated YH16.33 cells were not growth inhibited even in the presence of induced TNF- α , TGF- β , and GDF-10 cytokines observed in these cell cultures (Fig. 3). Future experiments are required to develop basic understanding of how engaging Ly-6A and the components of the antigen receptor complex, either alone or in collaboration with the cytokine receptor(s) transduce a distinct set of signals with contrasting outcomes. In regard to TNF, it is intriguing that TNF- α is known to induce either apoptosis/necroptosis or cell survival (MacEwan 2002; Rath and Aggarwal 1999). The opposing biological outcomes have been attributed to the two TNF- α receptors (TNFRI and TNFRII) and their downstream effectors causing either apoptosis/necroptosis (Rath and Aggarwal 1999) or cell survival, the latter being promoted by the activation of transcription factors, such as NF- κ B and c-Jun (Aggarwal 2000; Liu et al. 1996). Incomplete reversal of anti-Ly-6A antibody-induced growth inhibition and apoptosis by anti-TNF- α antibody suggests incomplete neutralization of TNF- α biological activity by the anti-TNF- α antibody and/or a contribution by other Ly-6A triggered cell death/signaling pathway that is distinct from TNF- α /TNFRI signaling. Taken together, our results suggest that the observed T cell growth inhibition and cell death is, in part, dependent on the presence of biologically active TNF- α . Signal modulatory effects of Ly-6A stimulation on TNFRI in the presence of its ligand TNF- α appear to be Ly-6A-specific, as these effects are not observed in cell cultures stimulated with anti-CD3 ϵ where TNF- α is detected, as well.

The Ly-6A protein is reported to interact with TGF- β receptors in mammary tumor cells and disrupt the GDF-10 (a TGF- β -like cytokine)-dependent inhibitory signaling to enhance tumorigenicity in breast cancer cells (Upadhyay et al. 2011). However, we did not detect significant amounts GDF-10 in anti-Ly-6A antibody-stimulated T cell cultures (Fig. 1), therefore suggesting its insignificant role in T cell growth inhibition. In addition, TGF- β , a known growth inhibitory cytokine (Moustakas and Heldin 2005; Shi and

Massagué 2003) was not abundantly detected (Fig. 1) and therefore is less likely to contribute to the observed growth inhibition, and apoptosis. In contrast, significantly high free active and total TGF- β and GDF-10 in the absence of T cell growth inhibition was detected in anti-CD3 ϵ -stimulated YH16.33T cells, as discussed above. In summary, GDF-10 and TGF- β are not produced by YH16.33T cells and therefore are less likely to play a biologically important role in Ly-6A-dependent growth inhibition.

Ly-6A is a negative regulator of T cell expansion and poses as an immune checkpoint inhibitor involved in the T cell clonal contraction phase of T cell response (Capozzoli et al. 2019). CD4⁺ T lymphocytes from Ly-6A-deficient mice show modest heightened proliferation rates compared to the controls (Stanford et al. 1997). Additionally, CD4⁺ T cells from Ly-6A transgenic mice displayed inhibited clonal expansion in response to a specific antigen (Henderson et al. 2002). The underlying mechanism of the growth inhibitory role of Ly-6A protein is unknown. Our findings with the clonal T cell line propose the role of TNF- α and not TGF- β and GDF-10 in the immune checkpoint inhibitory role of Ly-6A. TNF- α is a well-studied pro-inflammatory cytokine that plays a critical role in innate and adaptive immune responses observed in humans (Aggarwal et al. 2012), and its possible role in initiating and or propagating pathological conditions is observed (Kitcharoensakkul et al. 2020). Further research is required to understand the molecular mechanism of how Ly-6A, a GPI-anchored protein located in lipid rafts, modulates inhibitory signaling through the TNF- α receptor(s) as recently noted (Bamezai and Miwa 2022).

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Author Contributions AP performed experiments, analyzed the data, performed the statistical analysis and partly wrote the manuscript. SM performed experiments and edited the manuscript. AKB pre-designed the project, coordinated the project, analyzed the data generated by AP and SM, wrote the manuscript and edited the manuscript. All authors have read and approved the final manuscript.

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Data availability statement The raw datasets analysed to generate figures in the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no commercial or financial conflicts of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

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