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Inflammatory cytokines IL-17 and TNF-α up-regulate PD-L1 expression in human prostate and colon cancer cells

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Abstract

Programmed cell death protein 1 (PD-1) acts on PD-1 ligands (PD-L1 and PD-L2) to suppress activation of cytotoxic T lymphocytes. Interleukin-17 (IL-17) and tumor necrosis factor-α (TNF-α) are co-expressed by T helper 17 (T_H17) cells in many tumors. The purpose of this study was to test if IL-17 and TNF-α may synergistically induce PD-L1 expression in human prostate cancer LNCaP and human colon cancer HCT116 cell lines. We found that IL-17 did not induce PD-L1 mRNA expression, but up-regulated PD-L1 protein expression in HCT116 and LNCaP cells. TNF-α induced PD-L1 mRNA and protein expression in both cell lines. Neither IL-17 nor TNF-α induced PD-L2 mRNA or protein expression. IL-17 and TNF-α acted individually rather than cooperatively in induction of PD-L1 expression. IL-17 and/or TNF-α activated AKT, nuclear factor-κB (NF-κB), and extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathways in HCT116 cells, whereas only NF-κB signaling was activated in LNCaP cells. NF-κB inhibitor could diminish PD-L1 protein expression induced by IL-17 and/or TNF-α in both HCT116 and LNCaP cell lines. ERK1/2 inhibitor could also reduce PD-L1 protein expression induced by IL-17 and/or TNF-α in HCT116 cells, while AKT inhibitor could abolish PD-L1 protein expression induced by IL-17 and/or TNF-α in LNCaP cells. These results suggest that IL-17 and TNF-α act individually rather than cooperatively through activation of NF-κB and ERK1/2 signaling to up-

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Conflicts of interest

The authors declare no conflicts of interest.

regulate PD-L1 expression in HCT116 cells, while the two inflammatory cytokines act through activation of NF-κB signaling, in the presence of AKT activity, to up-regulate PD-L1 expression in LNCaP cells.

Keywords

Interleukin-17; Tumor necrosis factor-α; Programmed cell death protein 1 ligand 1; Prostate cancer; Colon cancer

1. Introduction

Cancer poses a major threat to public health worldwide due to its increasing prevalence and is a leading cause of deaths worldwide. In 2013, there were approximately 14.9 million incident cancer cases and 8.2 million cancer-related deaths [1]. Cancer is a chronic disease with multiple driver and passenger genes involved in the complicated pathogenesis [2]. In recent years, both laboratory and clinical studies revealed that immune escape is one of the most important mechanisms for cancer to avoid immune destruction and acquire resistance to anti-tumor drugs, which becomes a key barrier in cancer therapy [3,4]. Cancer immune escape mechanism involves many factors. One of these factors is programmed cell death protein 1 (PD-1) that acts on PD-1 ligands (PD-L1 and PD-L2) [5,6]. PD-1, encoded by the PDCD1 gene, is a cell surface receptor that belongs to the immunoglobulin superfamily and is expressed on T cells, B cells, monocytes, natural killer cells, dendritic cells, and regulatory T cells (Treg). PD-1 is considered as an immune checkpoint that inhibits activation of T cells to prevent autoimmunity and promote self-tolerance [7]. PD-L1 and PD-L2 belong to the membrane protein B7 family, also called B7-H1 and B7-DC, respectively. PD-L1, also known as cluster of differentiation 274 (CD274), is a 40 kDa type I transmembrane protein encoded by CD274 gene in humans. PD-L1 is expressed in a variety of cancer cells, such as prostate cancer, colorectal cancer, gastric cancer, lung cancer, melanoma, renal cell carcinoma, multiple myeloma, and leukemia [8–11]. Tumor-associated PD-L1 promotes apoptosis of cytotoxic T cells while enhancing survival of Treg cells, thus suppressing the anti-cancer immune system [12]. PD-1/PD-L1 signaling axis has been demonstrated to play a role in regulating tumor microenvironment of prostate cancer and colorectal cancer [13,14]. Lee et al [15] showed that 5% colorectal carcinomas exhibited high PD-L1 expression and 19% had elevated numbers of PD-1-positive tumor infiltrating lymphocytes. Gevensleben et al [10] found that 52.2% of 209 primary prostate cancer samples expressed moderate to high PD-L1 levels and PD-L1 positivity was prognostic for biochemical recurrence. However, how PD-L1 expression is regulated in colorectal cancer and prostate cancer remains elusive. It has been demonstrated that certain pro-inflammatory factors can up-regulate PD-L1 expression, including interferon-γ (IFN-γ), tumor necrosis factor – α (TNF-α), lipopolysaccharide, granulocyte-monocyte colony stimulating factor, vascular endothelial growth factor, interleukin-10 (IL-10), and IL-4, with IFN-γ being the most potent inducer [16]. Interleukin-17 (IL-17, also called IL-17A) is a key pro-inflammatory cytokine that has been shown to promote prostate and colon cancer development [17–20]. IL-17 and TNF-α have been shown to cooperate functionally to induce expression of down-stream genes [21]. Yet, it is unknown if IL-17 and TNF-α may

synergistically induce PD-L1 expression. The objective of the present study was to test if IL-17 and TNF-α may synergistically induce PD-L1 expression in human prostate cancer and colon cancer cells.

2. Materials and methods

2.1. Cell culture

Human colorectal cancer cell line HCT116 and human prostate cancer cell line LNCaP were purchased from the American Type Culture Collection (Manassas, VA, USA) and were free of mycoplasma contamination. HCT116 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Fisher Scientific) and 100 U/mL penicillin/streptomycin. LNCaP cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Fisher Scientific) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. Both cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C.

2.2. Reagents

Recombinant human IL-17 (also called IL-17A) and recombinant human TNF-α were obtained from Fisher Scientific. Akt inhibitor AZD5363 was obtained from Selleck Chemicals, Inc. (Houston, TX, USA). NF-κB inhibitor Bay11-7082 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), ERK1/2 inhibitor U0126 was obtained from Promega Corporation (Madison, Wisconsin, USA). The primary antibodies used were: rabbit anti-PD-L1 monoclonal antibody (ab205921, Abcam, Cambridge, MA, USA); rabbit anti-PD-L2 polyclonal antibody (SAB3500395, Sigma-Aldrich, St. Louis, MO, USA); rabbit anti-phospho-Akt^{Ser473} polyclonal antibody, rabbit anti-Akt polyclonal antibody, rabbit anti-phospho-IκBα polyclonal antibody, and mouse anti-IκBα polyclonal antibody(Cell Signaling Technology, Danvers, MA, USA); rabbit anti-phospho-ERK1/2 polyclonal antibody and rabbit anti-ERK1/2 polyclonal antibody (Santa Cruz Biotechnology); and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (#MAB374, Millipore, Billerica, MA, USA).

2.3. Quantitative real-time reverse transcription – polymerase chain reaction (qRT-PCR)

After the indicated time of treatment, cells were harvested for total RNA extraction using NucleoSpin RNA kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using PrimeScript™ reverse transcription kit (Takara, Mountain View, CA, USA). Human PD-L1, PD-L2, and GAPDH primers were obtained from Eurofins MWG Operon (Huntsville, AL, USA). The sequences are as follows: PD-L1, Forward: 5′-CTCAGGGTGACAGAGAGAGAAG-3′, Reverse: 5′-GACACCAACCACCAGGGTTT-3′; PD-L2, Forward: 5′-TGGCATTTGCTGACGCATTT-3′, Reverse: 5′-TGCAGCCAGGTCTAATTGTTTT-3′; GAPDH, Forward: 5′-CCACATCGCTCAGACACCAT-3′, Reverse: 5′-TAAAAGCAGCCCTGGTGACC-3′. qRT-PCR was performed in triplicates with an iQ5 iCycler and iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer′s protocols. Results were normalized to GAPDH levels using the formula Δ cycle threshold (Ct) = Ct of target gene - Ct of GAPDH. The mRNA level of

the control group (vehicle treatment) was used as the baseline; therefore, ΔΔCt was calculated using the formula ΔΔCt = ΔCt of target gene - ΔCt of the baseline. The fold change of mRNA level was calculated as fold = $2^{-\Delta\Delta Ct}$.

2.4. Western blot analysis

After the indicated time of treatment, proteins were extracted from the cells in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM sodium fluoride, 0.5% Igepal CA-630 [NP-40], 10 mM sodium phosphate, 150 mM sodium chloride, 25 mM Tris pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetic acid [EDTA], 1.2 mM sodium vanadate) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Equal amount of proteins was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline – Tween 20 (TBS-T) buffer (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween-20) for 1 hour and probed with the indicated primary antibodies overnight and then IRDye 800CW- or IRDye 680RD-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) for 1 hour. The results were visualized using an Odyssey Infrared Imager (LICOR Biosciences). For loading control, the membranes were stripped and probed for unphosphorylated proteins and/or GAPDH. Quantification of the Western blot signals was performed using the image analysis software of the Odyssey Infrared Imager system. The integrated density values of signals of targeted genes were normalized by those of GAPDH. The ratio indicates the relative level of target protein.

2.5. Statistical analysis

Statistical analysis was performed using SPSS (v17.0, SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD). Quantitative data were analyzed using analysis of variance (ANOVA, two-tailed) and Fisher's least significant difference (LSD) method. Statistical significance was reached with a P value less than 0.05.

3. Results

3.1. PD-L1, but not PD-L2, mRNA expression was up-regulated by TNF-α, but not by IL-17

Human colon cancer cell line (HCT116) and human prostate cancer cell line (LNCaP) were treated with IL-17, TNF-α, or a combination of both for 3 hours. qRT-PCR analysis found that IL-17 treatment alone did not affect expression of either PD-L1 or PD-L2 mRNA in HCT116 and LNCaP cells (Fig. 1). TNF-α treatment alone significantly increased expression of PD-L1, but not PD-L2, mRNA in both HCT116 and LNCaP cell lines (Fig. 1, $P < 0.001$). In HCT116 cells, a combination of IL-17 and TNF-α treatment further increased PD-L1 mRNA expression to a level that was significantly higher than TNF-α alone (Fig. 1A, $P = 0.038$). In contrast, the combination of IL-17 and TNF-α treatment increased PD-L1 mRNA expression to a level that was significantly lower than TNF-α alone in LNCaP cells (Fig. 1B, $P < 0.001$).

3.2. PD-L1, but not PD-L2, protein expression was up-regulated by both IL-17 and TNF-α

When HCT116 and LNCaP cells were treated with IL-17 and/or TNF-α for 8 hours, IL-17 or TNF-α alone or a combination of both significantly increased PD-L1, but not PD-L2, protein expression in both cell lines (Fig. 2). In HCT116 cells, the increased PD-L1 protein levels sustained up to 24 hours, whereas such increase in PD-L1 levels was diminished at 16 hours and disappeared at 24 hours in LNCaP cells (Fig. 2). Of note, although there was a slight further increase of PD-L1 protein levels in HCT116 cells treated with the combination of IL-17 and TNF-α compared to either cytokine alone, IL-17 and TNF-α did not appear to have any synergistic effects in induction of PD-L1 protein expression (Fig. 2).

3.3. IL-17 and TNF-α activated different signaling pathways in HCT116 and LNCaP cells

In HCT116 cells, IL-17, TNF-α, and a combination of both increased the phosphorylated AKT (p-AKT) levels in a time-dependent manner with the peak levels at 15 minutes, without affecting the AKT levels (Fig. 3A, C, and D). IL-17, TNF-α, and a combination of both increased the phosphorylated IκBα (p-IκBα) levels in a time-dependent manner with the peak levels at 5 minutes, which was accompanied with a decrease in IκBα levels (Fig. 3A, E, and F). TNF-α was significantly more effective in causing the changes in p-IκBα and IκBα levels than IL-17 (Fig. 3A, E, and F). IL-17, TNF-α, and a combination of both increased the phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2) levels in a time-dependent manner with the peak levels at 45 minutes, without affecting the ERK1/2 levels (Fig. 3B, G, and H). On the other hand, in LNCaP cells, IL-17, TNF-α, or a combination of both did not significantly change the levels of p-AKT or AKT (Fig. 4A to C), though the basal levels of p-AKT were high due to lack of phosphatase and tensin homolog (PTEN) expression in LNCaP cells [22]. IL-17, TNF-α, or a combination of both did not significantly change the levels of p-ERK1/2 or ERK1/2 (data not shown). Nevertheless, IL-17, TNF-α, and a combination of both increased the phosphorylated IκBα (p-IκBα) levels in a time-dependent manner with the peak levels at 5 minutes, which was accompanied with a decrease in IκBα levels (Fig. 4A, D, and E). TNF-α was significantly more effective in causing the changes in p-IκBα and IκBα levels than IL-17 (Fig. 4A, D, and E). Of note, the combination of IL-17 and TNF-α increased p-IκBα levels not as high as TNF-α alone, though IκBα levels were reduced similarly by TNF-α alone or the combination of IL-17 and TNF-α (Fig. 4A, D, and E).

3.4. Blockade of AKT, NF-κB, or ERK1/2 signaling pathways diminished PD-L1 protein expression induced by IL-17 and/or TNF-α

Since AKT, NF-κB, or ERK1/2 signaling pathways were activated in HCT116 cells by IL-17 and/or TNF-α, we tested if inhibitors of AKT (AZD5363), NF-κB (Bay11-7082), or ERK1/2 (U0126) signaling pathways could diminish PD-L1 protein expression induced by IL-17 and/or TNF-α. We found that AZD5363 only significantly reduced PD-L1 protein levels induced by IL-17, but not by TNF-α or a combination of IL-17 and TNF-α in HCT116 cells (Fig. 5A and C). In contrast, both U0126 and Bay11-7082 significantly diminished the increase of PD-L1 levels induced by IL-17, TNF-α, or a combination of both in HCT116 cells (Fig. 5A and C). On the other hand, in LNCaP cells, both Bay11-7082 and

AZD5363 significantly diminished the increase of PD-L1 levels induced by IL-17, TNF-α, or a combination of both (Fig. 5B and D).

4. Discussion

T helper 17 (T_H17) cells are a distinct group of CD4+ T cells that co-express IL-17 and TNF-α [23–25]. IL-17 and TNF-α-secreting T_H17 cells have been found to be enriched in colon tumors and prostate tumors [26,27]. Given that IL-17 and TNF-α have been shown to cooperate functionally to induce expression of down-stream genes [21], we investigated if IL-17 and TNF-α could synergistically induce PD-L1 expression in human prostate cancer and colon cancer cells. In the present study, we found that, although only TNF-α induced PD-L1 mRNA expression in human colon cancer HCT116 cell line and human prostate cancer LNCaP cell line, both IL-17 and TNF-α induced PD-L1 protein expression in HCT116 and LNCaP cells. We speculate that the increase of PD-L1 level is likely adequate to inhibit T cell function as shown in our recent study [28]. In that study, we found that estrogen up-regulated PD-L1 expression via PI3K/Akt signaling pathway and the increased PD-L1 expression suppressed interferon-γ and IL-2 expression in Jurkat cells and primary T cells [28]. Given that the amplitude of PD-L1 induction by IL-17 and TNF-α is comparable to or even higher than the amplitude induced by estrogen, we speculate that the two situations are comparable. Neither IL-17 nor TNF-α induced PD-L2 mRNA or protein expression, suggesting that PD-L2 expression may be regulated by different factors.

It is worth pointing out that IL-17 and TNF-α did not appear to have any synergy in inducing PD-L1 expression. Our findings showed that a combination of IL-17 and TNF-α increased PD-L1 mRNA levels to be higher than that induced by TNF-α alone in HCT116 cells, but the combination caused less increase of PD-L1 mRNA levels than TNF-α alone in LNCaP cells. The combined treatment increased PD-L1 protein expression to the levels similar to what was induced by TNF-α alone. We consistently observed that TNF-α was more effective than IL-17 in induction of PD-L1 expression. These findings suggest that IL-17 and TNF-α act individually rather than cooperatively in regulating PD-L1 expression in HCT116 and LNCaP cell lines.

In order to understand how IL-17 and TNF-α induce PD-L1 expression, we checked the signaling pathways that could be activated by IL-17 and TNF-α. We found that NF-κB signaling was activated as early as 5 minutes after treatment with IL-17 and/or TNF-α in both HCT116 and LNCaP cell lines. AKT signaling was activated 5 minutes after treatment with IL-17 and/or TNF-α in HCT116 cells, which became more dramatic at 15 minutes, while ERK1/2 signaling was activated 30 to 45 minutes after treatment with IL-17 and/or TNF-α in HCT116 cells. In contrast, AKT and ERK1/2 signaling pathways were not activated by IL-17 or TNF-α in LNCaP cells. These findings suggest that the responses to IL-17 and TNF-α are cell type-specific. To check if any of these signaling pathways is responsible for induction of PD-L1 expression, we used selective inhibitors to block each signaling pathway. We found that Bay11-7082 (NF-κB inhibitor) significantly diminished PD-L1 protein expression induced by IL-17 and/or TNF-α in both HCT116 and LNCaP cell lines. This finding is consistent with the finding that IL-17 and/or TNF-α activated NF-κB signaling as early as 5 minutes upon the treatment. AZD5363 is a pan-AKT inhibitor [29].

AZD5363 diminished PD-L1 protein expression induced by IL-17 and/or TNF-α in LNCaP cells, whereas it only diminished PD-L1 protein expression induced by IL-17 in HCT116 cells. This finding indicates that AKT signaling is not a major driver in inducing PD-L1 expression by TNF-α in HCT116 cells. Of note, Neither IL-17 or TNF-α activates AKT signaling in LNCaP cells. However, the basal level of AKT activity is high due to inactivation of PTEN in LNCaP cells [22]. It has been demonstrated that human immunodeficiency virus can activate PI3K/Akt to up-regulate PD-L1 expression [30]. We speculate that the basal level of AKT activity is required for IL-17 and TNF-α to induce PD-L1 expression in LNCaP cells, thus AZD5363 is able to diminish the effects of IL-17 and/or TNF-α. In addition, MEK/ERK1/2 inhibitor (U0126) also significantly diminished PD-L1 protein expression induced by IL-17 and/or TNF-α in HCT116 cells. Together, these findings suggest that IL-17 and/or TNF-α act through activation of NF-κB and ERK1/2 signaling to up-regulate PD-L1 expression in HCT116 cells, while the two cytokines act through activation of NF-κB signaling, in the presence of AKT activity, to up-regulate PD-L1 expression in LNCaP cells. Our findings are consistent with several previous studies showing that NF-κB plays a key role in regulating PD-L1 expression [31–34]. Previous studies have also demonstrated that activation of ERK1/2 signaling drives PD-L1 expression in melanomas and lung cancers [35,36]. Activation of ERK1/2 and Akt pathways has been shown to up-regulate PD-L1 expression in melanoma cells [37], colon cancers [38], and breast cancer [39]. The novelty of the present study is the finding that IL-17 and TNF-α act individually rather than cooperatively to up-regulate PD-L1 expression in human colon cancer HCT116 cell line and human prostate cancer LNCaP cell line, which has never been reported before.

5. Conclusion

The present study suggests that IL-17 and TNF-α act individually rather than cooperatively through activation of NF-κB and ERK1/2 signaling to up-regulate PD-L1 expression in HCT116 cells, while the two cytokines act through activation of NF-κB signaling, in the presence of AKT activity, to up-regulate PD-L1 expression in LNCaP cells. Given that IL-17 and TNF-α-secreting T_H17 cells have been found to be enriched in colon tumors and prostate tumors [26,27], our findings imply that T_H17 cells may create an immunosuppressive tumor microenvironment through up-regulating PD-L1 expression.

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Abbreviations

Ct	cycle threshold
DMEM	Dulbecco’s Modified Eagles Medium
ERK	extracellular signal-regulated kinase

FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IFN-γ	interferon-γ
IL-17	interleukin-17
NF-κB	nuclear factor-κB
PD-1	Programmed cell death protein 1
PD-L1	PD-1 ligand 1
PD-L2	PD-1 ligand 2
qRT-PCR	quantitative real-time reverse transcription – polymerase chain reaction
SD	standard deviation
T_H17	T helper 17
Treg	regulatory T cells
TNF-α	tumor necrosis factor-α

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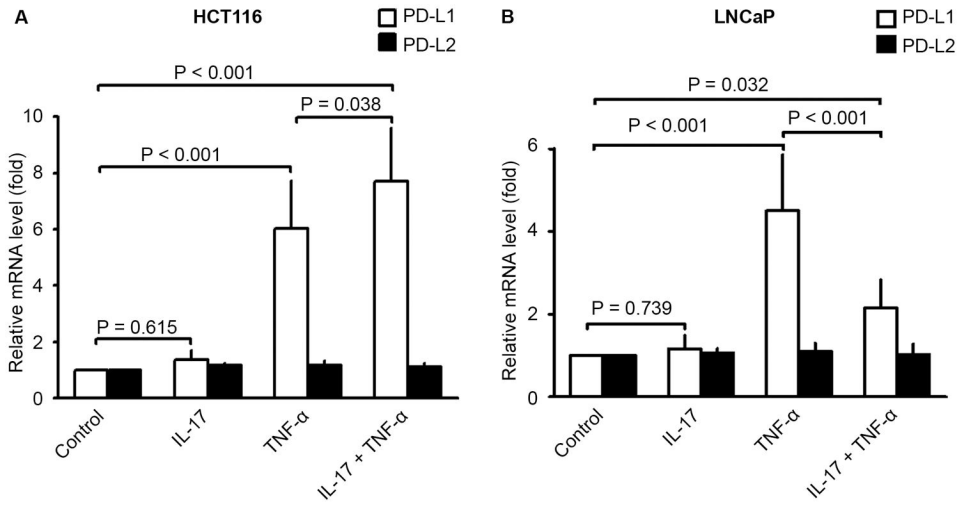

Fig. 1.

Effects of IL-17 and/or TNF-α on PD-L1 and PD-L2 mRNA expression in human cancer cell lines HCT116 and LNCaP. (A and B) The cells were treated with IL-17 (20 ng/ml), TNF-α (10 ng/ml), or a combination of both for 3 hours. PD-L1 and PD-L2 mRNA expression was determined by qRT-PCR analysis. Data were represented as means ± SD (error bars) of three independent experiments.

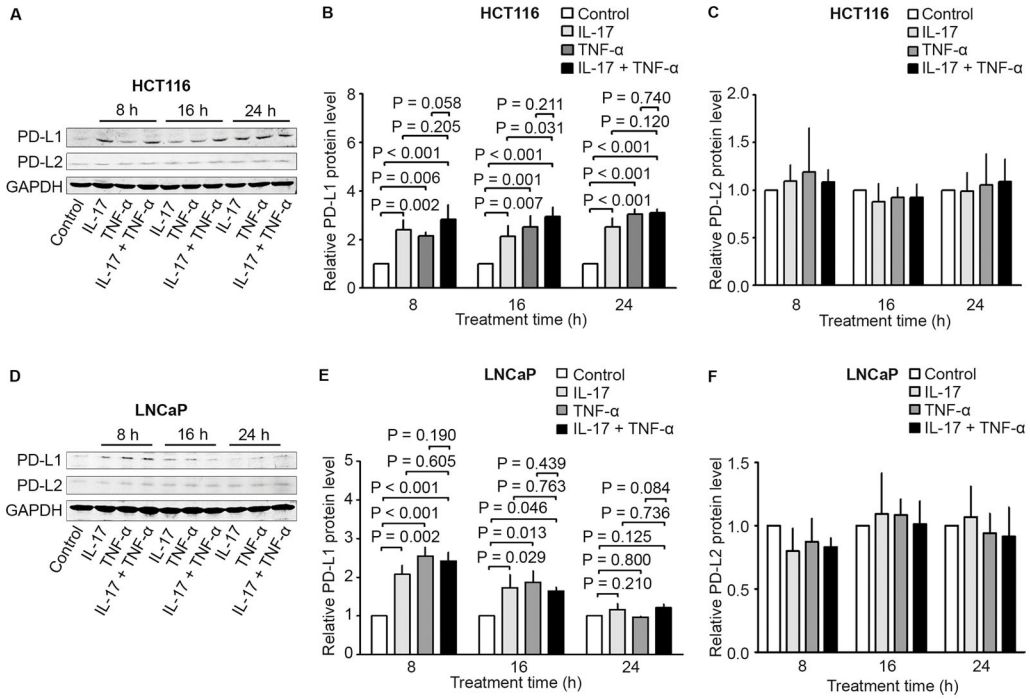

Fig. 2.

Effects of IL-17 and/or TNF-α on PD-L1 and PD-L2 protein expression in human cancer cell lines HCT116 and LNCaP. (A to C) HCT116 cells and (D to F) LNCaP cells were treated with IL-17 (20 ng/ml), TNF-α (10 ng/ml), or a combination of both for the indicated time. PD-L1 and PD-L2 protein expression was analyzed using Western blot analysis. GAPDH was probed for protein loading control (A and D). The relative protein levels were presented as the ratio of PD-L1/GAPDH or PD-L2/GAPDH (B–C and E–F), which was normalized against the ratio of the control group (arbitrarily designated as "1"). Data were represented as means ± SD of three independent experiments.

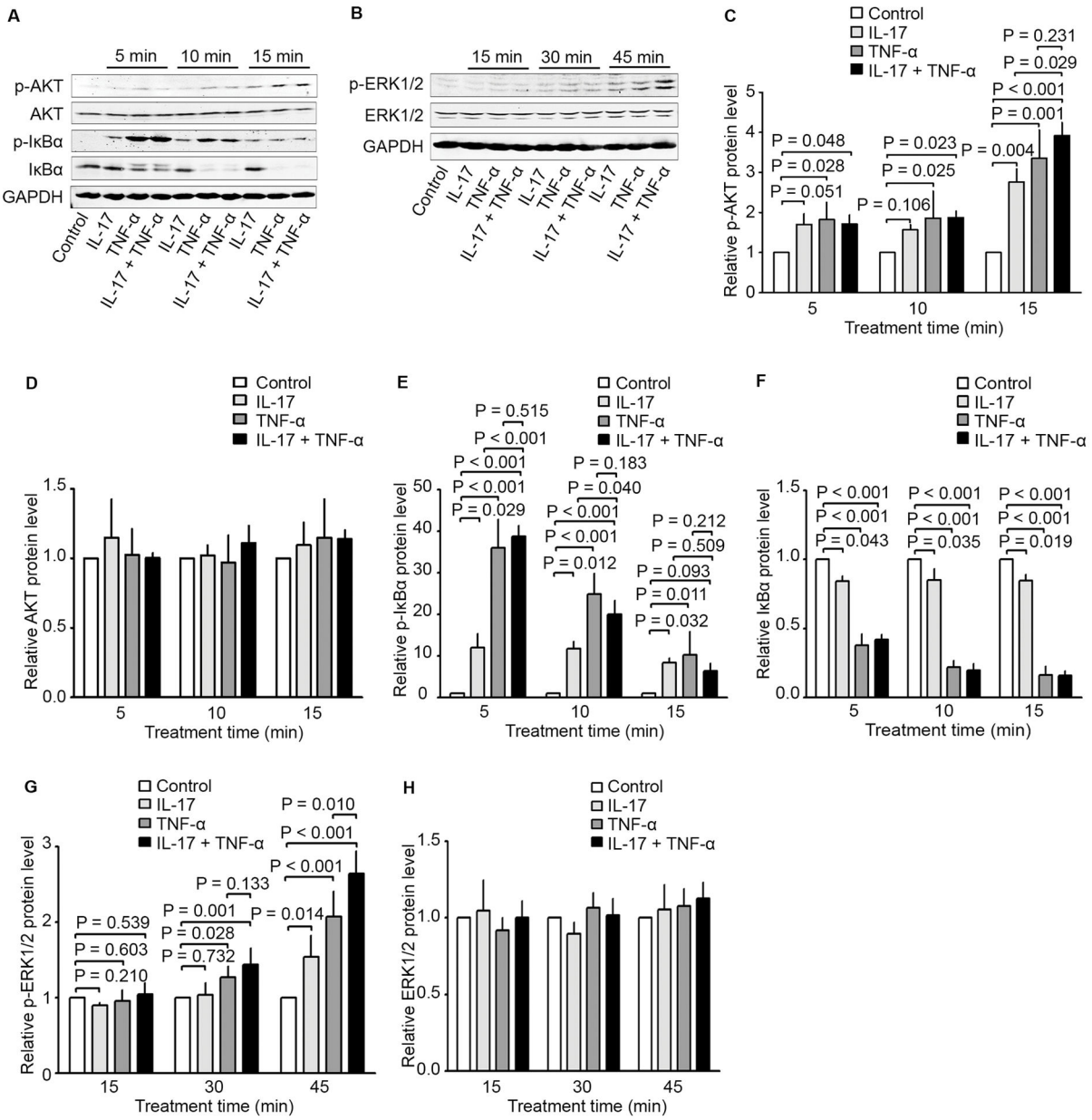

Fig. 3.

Effects of IL-17 and/or TNF-α on activation of signaling pathways in human colon cancer line HCT116. (A to H) HCT116 cells were treated with IL-17 (20 ng/ml), TNF-α (10 ng/ml), or a combination of both for the indicated time. Activation of Akt, IκBα, and ERK1/2 was assessed using Western blot analysis. GAPDH was probed for protein loading control (A and B). Relative protein levels were presented as the ratio of each protein divided by GAPDH, which was normalized against the ratio of the control group (arbitrarily designated as "1") (C to H). Data were represented as means ± SD of three independent experiments.

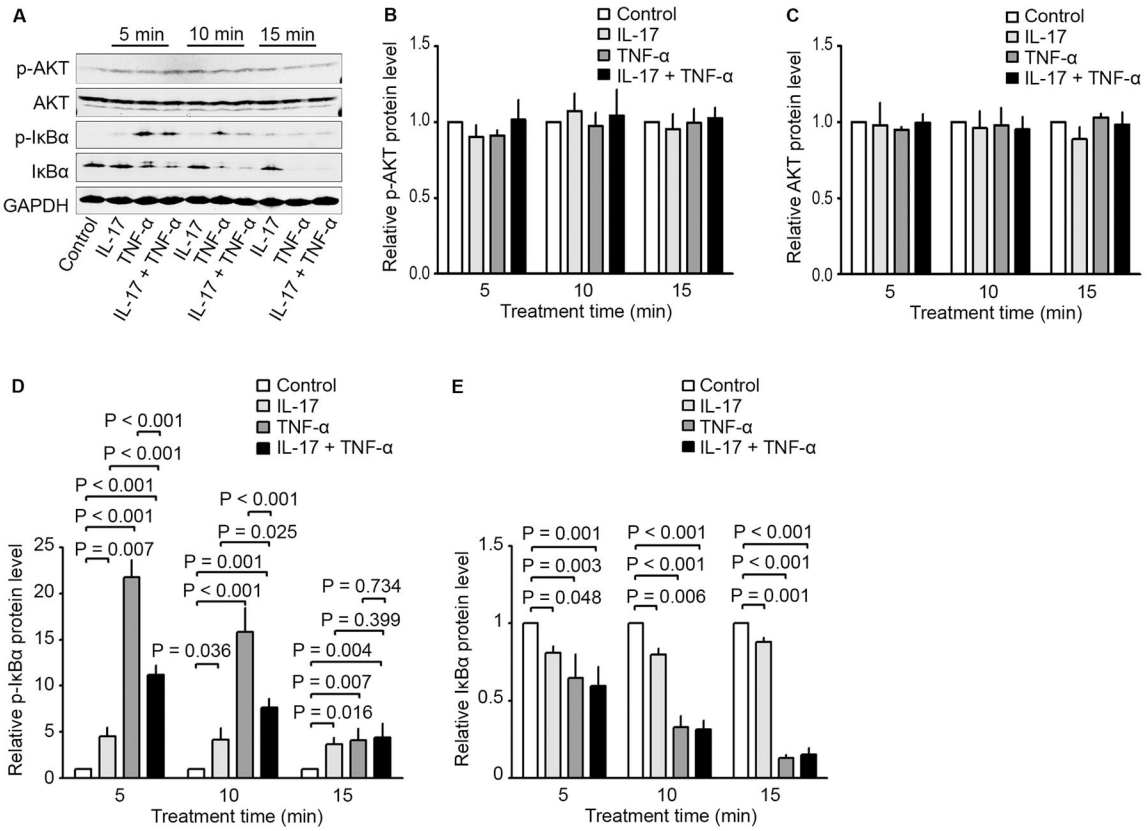

Fig. 4.

Effects of IL-17 and/or TNF-α on activation of signaling pathways in human prostate cancer line LNCaP. (A to E) LNCaP cells were treated with IL-17 (20 ng/ml), TNF-α (10 ng/ml), or a combination of both for the indicated time. Activation of Akt, IκBα, and ERK1/2 was assessed using Western blot analysis. GAPDH was probed for protein loading control (A). Relative protein levels were presented as the ratio of each protein divided by GAPDH, which was normalized against the ratio of the control group (arbitrarily designated as "1") (B to E). Data were represented as means ± SD of three independent experiments.

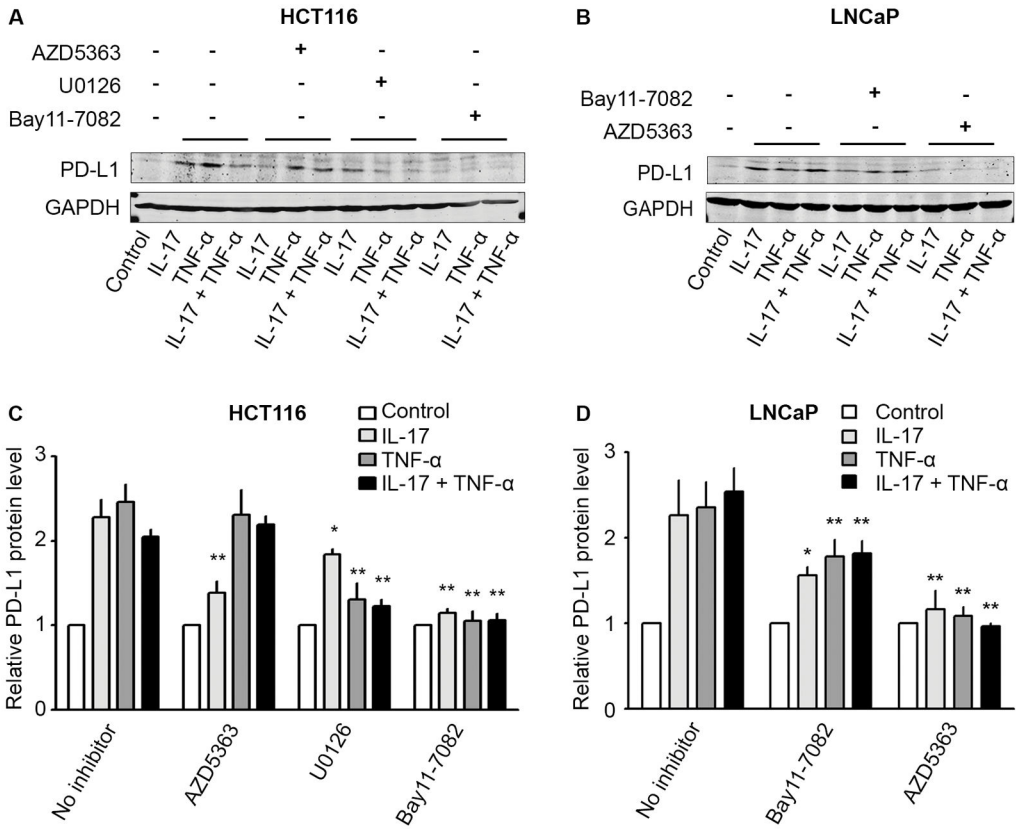

Fig. 5.

Effects of pan-Akt inhibitor (AZD5363), MEK/ERK1/2 inhibitor (U0126), and NF-κB inhibitor (Bay11-7082) on PD-L1 protein expression induced by IL-17 and/or TNF-α in human cancer cell lines HCT116 and LNCaP. (A and C) HCT116 cells and (B and D) LNCaP cells were first treated with AZD5363 (2 μM), U0126 (10 μM), or Bay11-7082 (5 μM) for 30 minutes. Then, HCT116 cells were treated with IL-17 (20 ng/ml), TNF-α (10 ng/ml), or a combination of both for 24 hours, while LNCaP cells were similarly treated for 8 hours. PD-L1 protein levels were analyzed using Western blot analysis. GAPDH was probed for protein loading control (A and B). Relative protein levels were presented as the ratio of PD-L1/GAPDH, which was normalized against the ratio of the control group (arbitrarily designated as "1") (C and D). Data were represented as means ± SD of three independent experiments. *P< 0.05 and **P< 0.01, compared to the corresponding group with IL-17 and/or TNF-α treatment in the absence of any inhibitor.