

Effects of 5-FU on DNA Synthesis and Cytotoxicity of Human Lymphocytes Induced by IL-2, TGF- β 3 and PGE₂

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Abstract. *Background:* Low 5-fluorouracil (5-FU) concentrations cause a significant increase in DNA synthesis in mitogen-activated human lymphocytes. *Materials and Methods:* We explored 2.5 μ M 5-FU-induced DNA synthesis by testing 5-FU activity in hypoxanthine-aminopterin-thymidine (HAT)-containing medium, and its effect on thymidylate synthase (TS) activity and CD25 expression in interleukin (IL)-2-activated human peripheral blood mononuclear cells (PBMCs) and the combined effects with prostaglandin E₂ (PGE₂) and transforming growth factor (TGF)- β 3. *Results:* The co-stimulatory effect of 2.5 μ M 5-FU on DNA synthesis was abrogated in HAT-cultured medium. 5-FU substantially reduced TS activity by 50% in IL-2-activated PBMCs. 5-FU combined with TGF- β 3 and PGE₂ did not alter their inhibitory effects on IL-2-activated natural killer cell cytotoxicity, but substantially affected increased DNA synthesis of cells cultured in IL-2 and co-cultured with 10 ng/ml TGF- β 3 and 10 μ M PGE₂. *Conclusion:* Low 5-FU concentrations increase DNA synthesis in lymphocytes and exert a co-stimulatory activity on TGF- β 3 and PGE₂ modulation of IL-2-activated lymphocytes.

5-Fluorouracil (5-FU) is a pyrimidine antimetabolite frequently used in the treatment of colorectal carcinoma and other malignancies of the digestive tract (1-3). In tumor cells, 5-FU acts on thymidine synthesis by regulating both the *de novo* pathway via the inhibition of thymidylate synthase (TS) (4) and the salvage pathway via thymidine kinase-1 (TK-1) (5). In addition to its effects on tumor cells, 5-FU exhibits inhibitory activities on various immune responses, including natural killer (NK) cell-mediated cytotoxicity (6,7) and mitogen-induced activation of lymphocytes (8). In previous experiments (9) which involved human peripheral blood lymphocytes from

healthy donors, we employed the ³H-thymidine incorporation assay and demonstrated that concentrations of 250-2,500 μ M of the drug substantially reduced DNA synthesis in culture following lymphocyte activation with interleukin (IL)-2, phytohemagglutinin A (PHA) and pokeweed mitogen (10). At the lower concentration of 2.5 μ M, however, 5-FU exhibited a co-stimulatory activity on mitogen-stimulated lymphocytes which was manifested by an substantial increase in DNA synthesis, as well as an increase in the percentage of cells entering the S-phase (9). These findings were unique to 5-FU since other DNA antimetabolites, including doxorubicin, 6-mercaptopurine and cyclophosphamide, failed to exhibit comparable effects (9). Other lymphocyte regulatory molecules which act as immunomodulators are prostaglandin E₂ (PGE₂) and transforming growth factor (TGF)- β . PGE₂ selectively affects CD45R0 T-cells (11), inhibits activated lymphocytes proliferation, IL-2 and interferon (IFN)- γ production by T helper (Th)1 clones (12), and reduces both *in vitro* and *in vivo* cytotoxic activities of NK cells (13). Furthermore, PGE₂ was shown to cause inactivation of leukocyte-specific protein tyrosine kinase (lck) and to reduce phosphorylation of ZAP70 which leads to cell cycle arrest in CD4⁺ lymphocytes associated with up-regulation of the cyclin-dependent kinase inhibitor p27 (14). TGF- β inhibits PHA- and IL-2-stimulated human peripheral blood lymphocytes (15, 16) via down-regulation of IL-2-mediated tyrosine phosphorylation (17). In addition, TGF- β directly suppresses NK (18) and T-cell cytotoxic activity via inhibition of the transcription of genes encoding for perforin and granzyme (19), reduces the secretion of tumor necrosis factor-alpha (TNF- α) and -beta (TNF- β) by lymphokine activated killer (LAK) cells (20), and inhibits DNA synthesis (21) via cell cycle arrest in late G₁ (22, 23). TGF- β also exhibits immunoenhancing effects on cytotoxic T lymphocytes *in vitro* (24).

In the present study, we extended our evaluation of the effect of 2.5 μ M 5-FU on DNA synthesis pathways by inhibiting *de novo* DNA synthesis using hypoxanthine-aminopterin-thymidine (HAT) medium, by measuring TS activity, and by assessing its effect on regulatory activities of PGE₂ and TGF- β in IL-2-activated human lymphocytes.

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Materials and Methods

Preparation of peripheral blood mononuclear cells (PBMCs). Twenty milliliters of heparinized blood obtained from healthy volunteers were diluted 1:1 in Hanks balanced salt solution (HBSS), pH 7.2. The blood was then layered on a 10-15 ml density-gradient solution (1.077 g/cm³, Ficoll; Pharmacia, Uppsala, Sweden) and spun at 550 xg for 15 min. The cells that had accumulated at the interphase between the plasma and Ficoll layers were collected, washed twice in HBSS, counted in 0.2% trypan blue solution to establish the percentage of viable cells, and resuspended at the desired density in enriched culture medium (CM) as described elsewhere (25).

5-FU, IL-2, PGE₂, TGF- β 3 and culture medium. 5-FU (50 mg/ml) was purchased from ABIC, Netanya, Israel. Human recombinant IL-2 (Cetus, Emeryville, CA, USA) has a specific activity of 3 \times 10⁶ IU/mg protein and endotoxin levels below 0.5 ng/mg recombinant IL-2. HAT medium (\times 10 concentration, Biological Industries, Beit-Haemek, Israel) was diluted 1:10 in enriched CM which was prepared as previously described (25). PGE₂ (Sigma, St. Louis, MO, USA) and TGF- β 3 (Biological Industries) were employed at 1-100 μ M and 0.1-100 ng/ml, respectively.

Generation of LAK cells and cytotoxicity measurement. LAK cells were generated as described elsewhere (25). Briefly, 2 \times 10⁶/ml PBMCs in CM were incubated for 3 days with 1,000 units/ml recombinant IL-2 in the presence or absence of 5-FU at different concentrations. The cytotoxic activity of LAK cells was measured by employing a 4-hour ⁵¹Cr-release assay where 10⁴/well of ⁵¹Cr-labeled NK-resistant Daudi tumor cells (Kindly provided by Dr. Steven Rosenberg, NCI, Bethesda, USA) served as targets. Four different effector-to-target ratios were employed in triplicate for each sample.

Calculation of % lysis and lytic units. Percent lysis was calculated according to the following formula:

$$\frac{[\text{mean cpm of experimental sample}] - [\text{mean cpm of spontaneous lysis}]}{[\text{mean cpm of target maximal lysis}] - [\text{mean cpm of spontaneous lysis}]} \times 100$$

where maximal lysis was achieved by incubating tumor targets in the presence of 0.1 N HCl, whereas spontaneous lysis was measured with targets incubated in CM alone. The spontaneous lysis in all the experiments did not exceed 20%. In all experiments, the cytotoxic activity is presented as lytic units (LU), which is defined according to the number of effector cells per 10⁶ IL-2-activated PBMC that lyses 30% of 10⁴ targets in a 4-hour ⁵¹Cr-release assay (25).

Proliferation assay. The proliferation assay was carried out as described in (25). PBMCs at 2 \times 10⁵/0.2 ml CM/well were incubated for 72 h in a 96-well round-bottom microplate with 10³ units/ml IL-2 in the presence or absence of different concentrations of 5-FU, after which 2 μ Ci/20 μ l/well [³H]thymidine (Sigma) were added to each well for the last 6 h of culture. The cells were then harvested and the radioactivity was determined by a liquid scintillation counter (LKB, Mt. Waverley, Australia).

Phenotype analysis. PBMCs were incubated for 72 h with 1,000 units/ml IL-2 in the presence or absence of 2.5 μ M of 5-FU. Cells were then harvested, washed and incubated for 60 min with fluorescent mouse anti-human CD25 monoclonal antibody (Becton Dickinson, San Jose, CA, USA) and then analyzed on a FACS Caliber (Becton Dickinson).

Cell cycle measurements. IL-2-activated PBMCs in the presence or absence of 5-FU were collected after 72 h and 96 h of incubation, washed twice in HBSS, and 10⁶ cells were exposed for 1 min to 50 μ l 1% triton followed by incubation for 1 min with 50 μ l of 1 mg/ml propidium iodide (PI). The cells were then analyzed by a flow cytometer.

Thymidylate synthase (TS) catalytic activity assay. TS was employed according to (26). Briefly, [5-³H]-2'-deoxyuridine was added to cells 72 h after the beginning of incubation. After a total incubation time of 21 hours, 450 μ l of the aqueous phase containing ³H₂O, was transferred to a scintillation vial and the radioactivity counted. Results are presented as cpm which correlates with TS activity.

Statistical analysis. Statistical analysis was performed by a two-tailed Student's *t*-test.

Results

Effect of 5-FU on de novo and salvage thymidine synthesis. In previous experiments, we used the ³H-thymidine incorporation assay and demonstrated that 2.5 μ M 5-FU substantially increased thymidine synthesis in IL-2-activated lymphocytes. Since thymidine synthesis was able to be carried out by both the *de novo* and salvage pathways, we tested the effect of 5-FU in PBMCs cultured in HAT medium, which blocks the *de novo* synthesis via the inhibition of dihydrofolate reductase. As illustrated in Figure 1, 5-FU, as previously shown, increased thymidine synthesis from 3,102 cpm (IL-2 alone) to 5,640 cpm in HAT-free medium. When cells were cultured in HAT medium, however, the stimulatory effect of 5-FU was completely abrogated (*i.e.*, 5,056 cpm in IL-2 vs. 3,605 in IL-2+5-FU cultures). These results indicate that 5-FU acts via the *de novo* thymidine synthesis pathway.

TS activity in lymphocytes following activation with IL-2 in the presence of 5-FU. TS is a major enzyme involved in the *de novo* synthesis of thymidine. Since we had shown in the previous experiment that the effect of 2.5 μ M 5-FU on DNA synthesis was carried out via the *de novo* pathway, we proceeded to test the effect of 5-FU on TS activity in IL-2-activated lymphocytes. As shown in Table I, IL-2 increased TS activity from 2,810 cpm in cells incubated in CM alone to 14,038 cpm. The addition of 5-FU to IL-2 cultures, however, reduced TS activity to 7,192 cpm. These findings demonstrate that the stimulatory effect of 2.5 μ M 5-FU on thymidine synthesis was not carried out by TS.

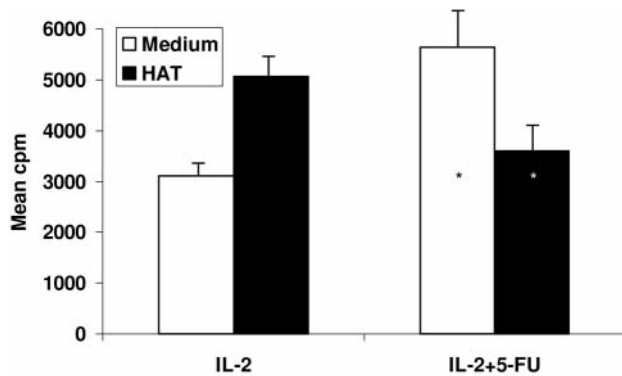


Figure 1. The effect of 5-FU on IL-2-induced DNA synthesis in cells incubated in HAT medium. PBMCs were incubated for 72 h in HAT medium with or without 1,000 U/ml IL-2, and 2.5 μ M 5-FU. DNA synthesis was then measured using the 3 H thymidine incorporation assay as described in the Materials and Methods section. * $p < 0.05$ Compared to IL-2 alone.

Effects of the combined treatment with IL-2 and 5-FU on the expression of IL-2 receptors on lymphocytes. Interaction of IL-2 with the high affinity IL-2 receptor CD25 induces tyrosine phosphorylation which leads to cell cycle progression of activated T cells from G₁-to S-phase (23). We therefore evaluated the effect of 2.5 μ M 5-FU on the expression of CD25 molecules in IL-2-activated lymphocytes. As shown in Table II, a small percentage of cells (2.7%) showed low CD25 intensity (MFC=33) following incubation without IL-2, which was not altered following the addition of 5-FU (MFC=34.2). When cells were incubated in IL-2, two populations were observed, one that expressed a low density of CD25 (CD25^{low}, MFC=56.7) and another that expressed a high density of CD25 (CD25^{high} MFC=223.8). The addition of 5-FU had minimal effect on both the intensity (MFC=53.3) and percentage (8.3%) of CD25^{low} cells but it substantially reduced CD25^{high} population (MFC=188.4).

Effects of FU on PGE₂ and TGF β 3 modulation of IL-2-induced cytotoxicity. We have previously shown that 2.5 μ M 5-FU had minor effects on the generation of activated NK cytotoxicity following incubation in IL-2. Since TGF- β and PGE₂ regulate NK activity (13, 18), we analyzed the combined effects of 5-FU, TGF- β 3 and PGE₂ on IL-2-activated NK cytotoxicity. As shown in Table III, 2.5 μ M 5-FU had no effect on IL-2-induced NK cytotoxicity (13.3 vs. 13.0 LU and 26.7 vs. 27.0 LU in experiment 1 and 2, respectively). The addition of TGF β 3 to IL-2 reduced NK cytotoxicity in both experiments, whereas the addition of PGE₂ to IL-2 reduced NK cytotoxicity to a lesser extent in both experiments. The addition of 2.5 μ M 5-FU to IL-2+TGF- β 3 further reduced NK cytotoxicity while the addition of 2.5 μ M 5-FU to IL-2 + PGE₂ cultures had minimal effect on NK cytotoxic activity, except at 50 E:T ratio ($p < 0.05$).

Table I. 5-FU reduces thymidylate synthase activity in lymphocytes incubated with IL-2 and PGE₂. PBMCs were incubated for 72 h in the presence of 2.5 μ M 5-FU and IL-2, after which the cells were harvested and tested for thymidylate synthase activity as described in the Materials and Methods section.

Treatment	Mean cpm	% Inhibition	p-Value
CM	2.810 \pm 1.067		
5-FU	4.010 \pm 890		NS*
IL-2	14.038 \pm 886		
IL-2+5-FU	7.192 \pm 1.303	49**	<0.01**

*Compared to CM; **compared to IL-2.

Table II. Effect of 5-FU on the expression of IL-2 receptors (CD25) on IL-2-activated lymphocytes. PBMCs were cultured for 72 h with/without IL-2 and 2.5 μ M 5-FU. The cells were then collected, incubated with anti-CD25-FITC-labeled antibody and analyzed as described in the Materials and Methods section.

Treatment	CD25 expression			
	Low MFC (low)		MFC (high)	
	MFC	% cells	MFC	% cells
CM	33.4	2.7		
5-FU	34.2	2.5		
IL-2	56.7	8.8	223.8	3.5
IL-2+5-FU	53.3	8.3	188.4	3.4

CM, Culture medium alone.

5-FU increases IL-2- and TGF- β 3-induced DNA synthesis. As shown in previous studies, 5-FU at a concentration of 2.5 μ M increased DNA synthesis in IL-2-activated human lymphocytes by up to 200% (9). In the present experiment, we further analyzed the combined effect of 2.5 μ M 5-FU, IL-2 and different concentrations of TGF- β 3 and PGE₂ on DNA synthesis. As illustrated in Figure 2, TGF- β 3 at concentrations of 1, 10 and 100 ng/ml significantly increased IL-2-induced DNA synthesis. The addition of 2.5 μ M 5-FU increased DNA synthesis significantly in cells treated with 1, 10 and 100 ng/ml TGF- β 3. When added to IL-2 cultures, PGE₂ significantly reduced DNA synthesis at 10⁻⁵ and 10⁻⁴ PGE₂, respectively (Figure 2B). The addition of 2.5 μ M 5-FU increased DNA synthesis significantly at 10⁻⁶ M PGE₂ + IL-2.

5-FU does not alter the effects of TGF- β 3 on the cell cycle following IL-2 activation. Incubation of PBMCs in the presence of IL-2 and 2.5 μ M 5-FU substantially increased the percentage of lymphocytes entering the S-phase of the cell cycle (9). Since 5-FU increased TGF- β 3-induced DNA synthesis following IL-2 activation, we tested the combined effect of these molecules on cell cycle phases. As shown in

Table III. Effect of 5-FU on the generation of IL-2-activated NK cytotoxicity in the presence of TGF- β 3 and PGE₂. PBMCs were cultured for 72 h in the presence of 1,000 U/ml IL-2, 10 ng/ml TGF- β 3 and 10 μ M PGE₂. The cells were then collected and tested at the indicated effector to target ratios against ⁵¹Cr-labeled Daudi tumor cells which served as targets. Results are presented as percent lysis and lytic units (LU) as described in the Materials and Methods section.

	Experiment 2				Experiment 1			
	% Lysis			LU	% Lysis			LU
E:T ratio	20	4	0.8		50	10	2	
IL-2	45 \pm 9	21 \pm 1	6 \pm 1	13.0	84 \pm 5	53 \pm 3	16 \pm 1	27.0
IL-2+5-FU	48 \pm 6	19 \pm 4	5 \pm 1	13.3	77 \pm 1	50 \pm 3	16 \pm 1	26.3
IL-2+TGF- β 3	35 \pm 2	10 \pm 0*	2 \pm 1*	6.9	56 \pm 4*	26 \pm 2*	8 \pm 0*	8.0
IL-2+PGE ₂	36 \pm 1	9 \pm 0*	1 \pm 1*	7.1	62 \pm 5*	31 \pm 4*	5 \pm 1*	10.1
IL-2+TGF- β 3+5-FU	31 \pm 2	9 \pm 2*	0 \pm 0*	5.9	43 \pm 7*	17 \pm 1*	6 \pm 1*	4.2
IL-2+PGE ₂ +5-FU	32 \pm 2	9 \pm 0*	1 \pm 0*	6.0	46 \pm 4*	31 \pm 1*	9 \pm 1*	10.4

*Statistically significant ($p < 0.05$) compared to IL-2 alone at the corresponding E:T ratio and between IL-2+PGE₂ and IL-2+PGE₂+5-FU at 50 E:T ratio.

Table IV, TGF- β 3 substantially increased cell arrest in the G₀/G₁-phase. The addition of 5-FU did not alter the TGF- β 3-induced increase of cells in the G₀/G₁-phase. The addition of 2.5 μ M 5-FU to IL-2 increased the percentage of cells entering the S-phase whereas TGF- β 3+IL-2 substantially reduced the percentage of cells in the S-phase; the addition of 5-FU to TGF- β 3+IL-2 did not alter this inhibitory effect. Furthermore, TGF- β 3 reduced the percentage of cells entering the G₂/M-phase from 4.1% (IL-2 alone) to 2.76%, while the further addition of 5-FU reduced the percentage of cells in this phase.

Discussion

We had previously demonstrated that 2.5 μ M 5-FU caused a substantial increase in DNA synthesis following activation of human lymphocytes (10). We also showed that this effect was unique to 5-FU since other antimetabolites, including doxorubicin, cyclophosphamide and 6-mercaptopurine (9), failed to exhibit an equivalent stimulatory activity on DNA synthesis. In the present study, we extended our investigation and further demonstrated that 5-FU increased DNA synthesis *via* the *de novo* pathway, since this effect was abrogated in HAT-containing medium (Table I). Nevertheless, TS, which is a key enzyme in the *de novo* thymidine synthesis (4), was substantially reduced by 2.5 μ M 5-FU (Table II), thus excluding the possibility that 5-FU exerted its activity *via* this enzyme. Others (5) have shown that 5-FU affected salvage DNA synthesis by increasing the activity of TK-1. This effect, however, was achieved *in vitro* at a substantially higher concentration of the drug (390 μ M) than the one employed in our study (2.5 μ M). The mechanism by which 5-FU regulates DNA synthesis is still unknown. We have shown that 2.5 μ M 5-FU substantially reduced the density of IL-2 receptors in the CD25^{high} (Table II), which were shown by others to initiate tyrosine phosphorylation and progression

of cells from the G₁-to the S-phase (23, 27). One possibility is that 2.5 μ M 5-FU acts *via* the activation of other enzymes, such as methionine synthase, which are involved in *de novo* DNA synthesis. Alternatively, based on our previous studies which indicate that 5-FU increased the percentage of cells in the S-phase (9) (Table IV), it is possible that 5-FU acts on molecules such as cyclin A, cyclin E and cyclin-dependent kinase 2, which promotes the G₁/S-phase shift transition during the cell cycle (28). Experiments designed to reveal a possible involvement of these molecules in 5-FU regulation of DNA synthesis are currently underway in our laboratory.

The effects of 2.5 μ M 5-FU on the regulatory activities of TGF- β 3 and PGE₂ on activated lymphocytes were also analyzed. Our findings confirm those of others (13, 18) by showing that both TGF- β 3 and PGE₂ substantially reduced NK cytotoxic activity following IL-2 activation (Table III). The addition of 2.5 μ M 5-FU to both PGE₂ and TGF- β 3 further reduced NK cytotoxic activity, similar to the effects of 5-FU on the generation of NK cytotoxicity in the presence of IL-2 alone (10). When the effect of 2.5 μ M 5-FU on DNA synthesis in the presence of 1-100 ng/ml of TGF- β 3 was evaluated, however, there was an increase of up to 48% in DNA synthesis in IL-2-activated lymphocytes (Figure 2A). This effect was similar to the effect of TGF- β 3 on lymphocytes undergoing activation by staphylococcal enterotoxin (29) and to the increased generation of allospecific cytotoxic T lymphocytes by 10-40 ng/ml TGF- β 1 when added at the beginning of culture (24). The increase in DNA synthesis by TGF- β 3 was further enhanced by up to 121% following the addition of 2.5 μ M 5-FU (Figure 2A). This stimulatory effect of 5-FU and TGF- β 3 on IL2-induced DNA synthesis was not reflected in the cell cycle since both 5-FU and TGF- β 3 substantially reduced (by up to 90%) the percentage of cells in the S-phase (Table IV). A similar effect of 5-FU was noted when it was added to IL-2-activated cells in the presence of PGE₂.

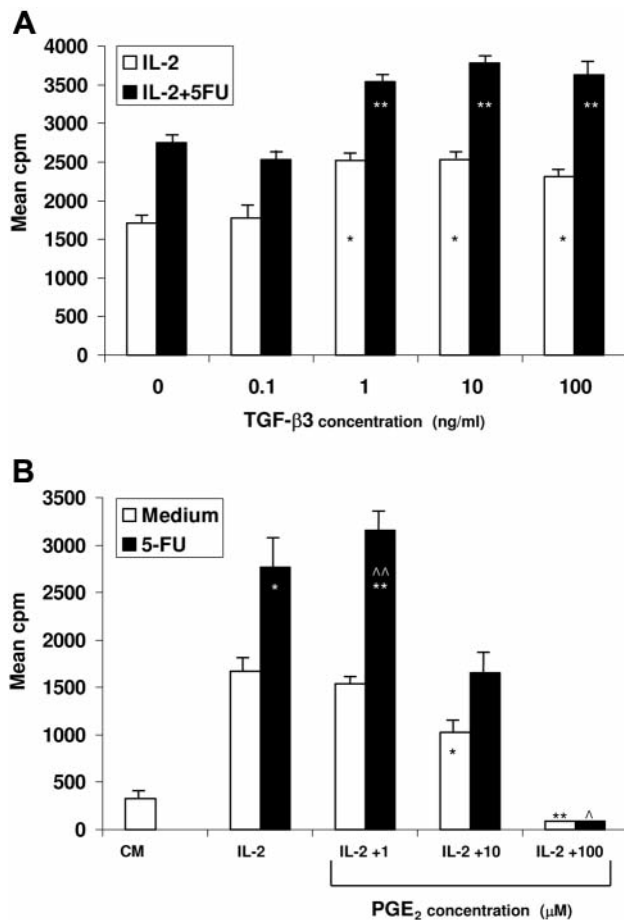


Figure 2. Modification by 5-FU of the effects of TGF-β3 and PGE₂ on IL-2-induced DNA synthesis. PBMCs were incubated for 72 h with 1,000 U/ml IL-2 and 2.5 μM 5-FU in the presence of either TGF-β3 (A) or PGE₂ (B). Cells were then tested for DNA synthesis using the ³H thymidine incorporation assay as described in the Materials and Methods section. The standard error of the mean (SEM) is presented for each column. A: **p*<0.05 Compared to IL-2 alone, ***p*<0.05 compared to the same culture without 5-FU; B: **p*<0.05 and ***p*<0.01 compared to IL-2 alone; Δ *p*<0.05 compared to IL-2 + 5-FU; ΔΔ *p*<0.05 compared to IL-2 + PGE₂ 1 μM.

Finally, our findings in this study showed that low concentrations of 5-FU modulated the activity of TGF-β3 and PGE₂ in cells activated by cytokines. Since these molecules are produced in cancer patients (30, 31), the possible effects of 5-FU on selected immune responses in these patients become matters of considerable interest. Studies aiming to evaluate the effects of 5-FU on lymphocytes isolated from cancer patients are currently being conducted in our laboratory.

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Table IV. 5-FU and TGF-β3 modulation of cell cycle and apoptosis in IL-2-activated lymphocytes. PBMCs were cultured for 72 h in the presence of IL-2 and TGF-β3, followed by a cell cycle analysis by a flow cytometer after incubation with propidium iodide.

Treatment	Cell cycle phase (% cells)			Apoptosis (% cells)
	G ₀ /G ₁	S	G ₂ /M	
IL-2	88.2	3.62	4.10	3.28
IL-2+5-FU	88.3	4.28	3.64	2.56
IL-2+TGF-β3	94.2	0.44	2.76	1.94
IL-2+5-FU+TGF-β3	94.9	0.44	2.36	1.84

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