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Restoration of Peripheral Intermediate and Classical Monocytes Expressing HLA-DR in Patients With Lung Adenocarcinoma After Platinum-Based Chemotherapy

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Abstract

Lung adenocarcinoma represents one of the lung cancer subtypes with major prevalence. Accumulating evidence indicates that the immune system plays an important role in the evolution of the neoplastic process; additionally, several reports suggest that chemotherapy has an immunomodulatory effect. In order to identify the peripheral subpopulations of leukocytes that may change after chemotherapy, we evaluated several peripheral immune subpopulations of monocytes and lymphocytes by multicolor flow cytometry. In addition, we also measured cytokines and growth factors on plasma in order to evaluate the pro-inflammatory context in patients with lung adenocarcinoma after chemotherapy. We found that HLA-DR$^+$ classical and intermediate monocytes were decreased in patients before chemotherapy, compared to controls. After chemotherapy, the relative percentage of those subpopulations was restored. In addition, interleukin 1β, interleukin 12, and interleukin 5 were increased after chemotherapy compared to prechemotherapy levels, while MIP-1β was decreased.

Keywords

lung adenocarcinoma, chemotherapy, immune cell subpopulations

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Abbreviation

IL, interleukin; NSCLC, non-small cell cancer; PD-1, programmed death ligand 1

Introduction

Lung adenocarcinoma is one of the most prevalent subtypes of non-small cell cancer (NSCLC); it has a poor prognosis, with a 5-year life expectancy of less than 30% when detected at an advanced stage (IIIB-IV),[1] which occurs in most cases.[1] The main therapeutic option for those patients who cannot undergo surgical resection is platinum-based chemotherapy.[2] Chemotherapy can improve the quality of life of patients with cancer and prolong their survival time, even in the most advanced clinical stage (stage IV).[3]

The immune system has the ability to detect and eliminate neoplastic cells. Some tumors express antigens that can be recognized by T CD8$^+$ cytotoxic lymphocytes,[4] and specific antibodies against tumor antigens can lead to the destruction of tumor cells.[5,6] However, in most clinical cases of cancer, the

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antitumor immune response is limited by the action of immune suppressor cells of lymphoid origin such as regulatory T cells ($CD4^+$, $CD25^+$, and $FoxP3^+$)[7] and programmed death ligand 1 ($PD\text{-}1^+$) cells.[8] Furthermore, some macrophages in the inflammatory infiltrate have a pro-tumor effect, promoting neovascularization and tumor metastasis.[9] Macrophages originate from circulating monocytes, which are a heterogeneous myelogenous population. Three subpopulations of monocytes have been described based on the expression of CD14 and CD16: classical ($CD14^{++}$, $CD16^-$), comprising 90% of circulating monocytes, intermediate ($CD14^{++}$, $CD16^+$), and nonclassical monocytes ($CD14^{Neg/+}$, $CD16^+$).[10,11] These subpopulations of monocytes exhibit different inflammatory, angiogenic, and phagocytic activity.[12]

The immunological parameters in neoplastic tissue and peripheral blood that have been associated with a poor prognosis in cancer include the increase in the expression of the PD-1 molecule in lymphocytes of neoplastic tissue[13,14] and in T cells of peripheral blood[8]; they also include an increase in the frequency of regulatory T cells[7] and HLA-$DR^{Low/neg}$ monocytes.[15-17] It has been recently reported that $CD14^+$ HLA-$DR^{Neg/Low}$ peripheral cells have immunosuppressive functions in patients with different types of cancer.[16]

Furthermore, it has been reported that cisplatin has a pleiotropic immunomodulatory effect. In addition to inhibiting mitosis, this antineoplastic drug increases the migration and proliferation of effector cells such as T $CD8^+$ lymphocytes specific for tumor antigens, as well as migration of macrophages and memory T cells.[18] Moreover, cisplatin increases the lytic activity of cytotoxic lymphocytes and the sensitivity of neoplastic cells to the action of cytotoxic cells; it also upregulates the expression of MHC/HLA class I molecules in macrophages.[18]

A recent study proposed the inclusion of systemic and local immunological parameters (Immunoscore) in the criteria used for cancer staging and to predict the outcome of chemotherapy.[19,20] Although several experimental studies have pointed out the importance of the immune system in the development of cancer,[21] there have been few studies focused on the usefulness of immune parameters as indicators of the evolution of patients with lung adenocarcinoma, one of the most prevalent types of lung cancer. The present study analyzed the ratio between activation and suppression in immune subpopulations of peripheral blood from patients with pulmonary adenocarcinoma, before and after the third cycle of mainly platinum-based chemotherapy. This was done with the aim of identifying changes in the activation and/or suppression of immune subpopulations of peripheral blood after chemotherapy. A panel of molecules was used to assess the pro-inflammatory context; the panel included cytokines, chemokines, and growth factors present in the plasma of patients and controls.

Methods

Ethics Statement

This study was approved by the Scientific and Bioethics Committee of the National Institute of Respiratory Diseases "Ismael Cosío Villegas." All participants provided written informed consent.

Patient Recruitment

A total of 9 patients with a diagnosis of lung adenocarcinoma were recruited among those attending the Oncology Pneumology Service and chemotherapy units of INER; 90% of them were female, with a mean age of 56.3 years (35/74). Patients with cardiac comorbidities, renal failure, and uncontrolled diabetes were discarded. The reference values of the populations under study were estimated by recruiting 9 matched healthy controls.

Sample Preparation

Five to 10 mL of peripheral blood from study participants was collected using the Vacutainer system (Becton Dickinson, NJ, USA). Subpopulations of interest were determined by flow cytometry in anticoagulated blood, as described in the following sections. The molecules of interest were measured in plasma recovered from the same blood samples. The plasma was obtained from anticoagulated peripheral blood, which was centrifuged at 1300*g* for 10 minutes at 25°C. The plasma collected was frozen at −80°C. Before use, plasma was centrifuged at 10 000*g* for 10 minutes at 4°C, to remove lipid components.

Cytometry Determinations

Human regulatory T cells were determined using a commercial kit (BioLegend, San Diego, CA). Mononuclear cells 1×10^6 were used; samples were stained according to the manufacturer's specifications with the following antibodies: CD4-PerCP, CD25-PE, and FOXP3-Alexa Fluor 488.

Subpopulations of monocytes and T CD4 and CD8 lymphocytes were determined by staining whole blood samples. The antibody panels used were titered and were validated using FMO controls, which were also used as gating controls. In addition, compensation controls were used. Each of the following antibody panels were added to 100 µL of anticoagulated blood: T CD4 lymphocytes (CD4 FITC, HLA-DR PE, CD3APC/Cy7, and CD279 APC), T CD8 lymphocytes (CD8 FITC, HLA-DR PE, CD3 APC/Cy7, and CD279 APC), and monocytes (CD16 Alexa Fluor 488, CD14 PE, HLA-DR PE/Cy7, and CD15 PerCP/Cy5.5). Samples were incubated for 20 minutes at room temperature, protected from light. Afterward, erythrocytes were lysed with a commercial solution (BioLegend) according to the manufacturer's instructions. Subsequently, samples were centrifuged at 350*g* for 5 minutes and washed with 2 mL of staining buffer. Finally, cells were washed and resuspended in 300 µL of staining buffer. All samples were acquired using an Accuri C6 flow cytometer (Becton Dickinson, San Jose, California; 2-2 configuration) and analyzed using the FlowJo X software (TreeStar, San Carlos, California). For human regulatory T cells, we used the 3-1 cytometry configuration.

Determination of Plasma Inflammatory Molecules

The concentration of inflammatory molecules was determined using the Luminex system (Bio-Plex Pro Human Cytokine 17-plex Assay kit; M5000031YV). The sample was processed according to the manufacturer's instructions and read using a Bio-plex 200 system (BioRad, CA). The results were analyzed using the Bioplex Manager software (v6.1).

Statistical Analysis

Statistical analysis was performed using the SPSS software (La Jolla, California). Continuous variables are expressed in medians $\pm$ standard error in the case of cell subpopulations and in means in the case of cytokine concentrations. We used the Shapiro-Wilk test to analyze the normality of all the variables. This test is designed for a small sample size with an n of up to 50. Then, nonparametrical variables ($P \leq .05$ on the Shapiro-Wilk test) were analyzed using the Wilcoxon test, as indicated in the tables and figures. The homogeneity of variances was confirmed by Levene test. Variables with normal distribution were analyzed using Student t test. Differences were considered significant when $P \leq .05$.

The data on the concentration of inflammatory molecules in plasma were divided into the following groups: pretreatment patients, posttreatment patients, and healthy controls. A t test for independent samples was used to compare the control group with patients before chemotherapy and patients after chemotherapy. A t test for dependent samples was used to compare the groups of patients before and after chemotherapy. On the determination of inflammatory molecules, we had missing values. We performed an analysis of missing values using SPSS. In the corresponding figure, the n value of each group is indicated. Differences were considered significant when $P \leq .05$. The survival rate of patients was analyzed using Kaplan-Meier curves over a period of 33 months.

Table 1. Clinical and Demographic Characteristics of Patients With Lung Cancer and Healthy Controls.

Parameter	Cases	Healthy Individuals
Number	9	9
Age, average (min-max)	56.3 (35-74)	54.8 (32-67)
Gender		
Male	2	2
Female	7	7
BMI	24.43 (18-32.4)	23.7 (20.7-27.0)
Exposure		
Smoking	1	4
Wood smoke	5	3
Biomass	1	0
Asbestos	4	1
Histologic type		
Lung adenocarcinoma	9	
Mutations		
EGFR positive	2	
ALK positive	0	
Kras positive	0	
Metastases		
Nodes	2	
Bone	4	
SNC	1	
Multiple	2	
Comorbidities or diseases		
Diabetes mellitus type 2 (DM2)	1	0
Systemic hypertension (SH)	1	1
CNS disease and DM2	1	0
Hyperthyroidism and SH	1	0
No comorbidity	5	
Initial APACHE score >25	4	
Initial APACHE score <25	5	
Initial chemotherapeutic regimens		
Platinum-based therapy	7	
Tyrosine kinase inhibitors (Gefitinib)	2	

Abbreviations: BMI, body mass index, min, minimum; max, maximum.

Results

The study group included 9 patients with a confirmed diagnosis of pulmonary adenocarcinoma, of which 78% were female and had a mean age of 56.3 (35/74) years. Their clinical characteristics are detailed in Table 1. Nine healthy individuals with similar characteristics of age and sex were recruited as controls (Table 1).

In order to estimate the patients' survival rate after treatment with chemotherapeutic drugs, Kaplan-Meier tests were performed during the study period. To estimate differences between the different chemotherapy regimens, the log-rank test was used and a χ^2 value = 2395, gl = 3, and $P \leq 0.495$ were obtained. The overall survival rate of the patients was 44% with an average survival of 13 months (Figure 1). No differences were found in survival between chemotherapy regimens.

Subpopulations of lymphocytes and peripheral monocytes associated with the activation or suppression of the immune system were analyzed, as well as a panel of inflammatory molecules. Regarding the populations of suppressor cells, there were no statistically significant differences in T regulatory cells ($CD4^+$, $CD25^+$, $FoxP3^+$) and $CD4^+$ $PD\text{-}1^+$ T cells between patients and controls (Table 2), but the proportion of $CD4^+$ $PD\text{-}1^+$ T cells was higher in patients after treatment with chemotherapy, from 0.29% to 2.42%, representing a fold increase of 8.3 (Figure 2).

Monocyte subpopulations were analyzed based on the expression profile of CD14 and CD16 as follows: classical monocytes ($CD14^{++}$, $CD16^-$), intermediate monocytes ($CD14^{++}$, $CD16^+$), and nonclassical monocytes ($CD14^{Neg/+}$, $CD16^{++}$). Patients before chemotherapy showed a 2.77-fold increase in the relative percentage of intermediate monocytes compared to control individuals (5.03% vs 13.96%, $P \leq .017$; Figure 3). No statistically significant differences were found in the proportion of monocyte subpopulations before and after chemotherapy (Table 3). Subsequently, the expression of HLA-DR was evaluated on monocyte subpopulations.

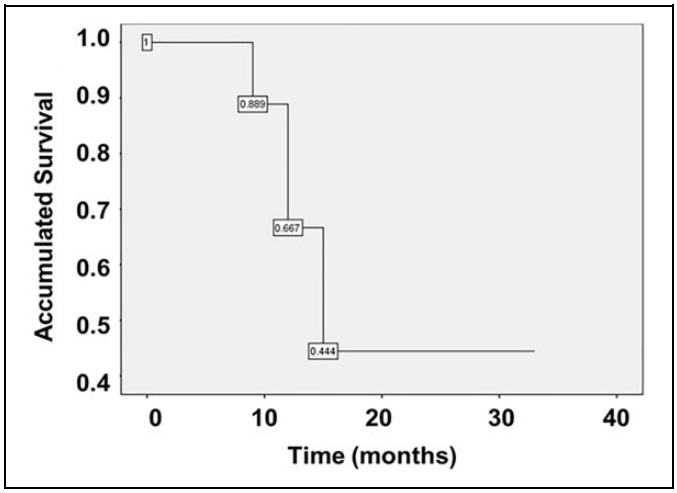

Figure 1. Kaplan-Meier survival curve of patients with lung adenocarcinoma.

The expression of HLA-DR in the monocyte population is very heterogeneous; for this reason, we first analyzed the percentage of HLA-DR$^-$ and HLA-DR$^+$g cells (Supplemental Figure 1). After gating HLA-DR$^+$g cells, we analyzed whether between study groups, HLA-DR-positive cells had differences between positive and highly positive cells. In the analysis of this parameter, cells were divided into positive and highly positive for HLA-DR; the threshold was set at the MFI (1×104). Thus, this study reports HLA-DR$^-$, globally positive HLA-DR cells (g), HLA-DR$^+$, and HLA-DR^{++}.

The proportion of HLA-DR$^-$ classical monocytes was found to be moderately increased in patients before chemotherapy in relation to control individuals, from 22.7% ($\pm$2.95%) to 28.05% ($\pm$3.85%), representing 1.2-fold increase; however, these differences were not statistically significant. After chemotherapy, the proportion of HLA-DR$^-$ classical monocytes decreased from 28.05% ($\pm$3.85%) to 11.38% ($\pm$3.78%), $P \leq .010$ (Table 3); similarly, the proportion of HLA-DR$^-$ intermediate monocytes was increased in patients before chemotherapy in relation to control individuals, from 9.77% ($\pm$3.23%) to 18.29% ($\pm$3.13%), which represents an increase of 1.87 times. Subsequent to chemotherapy, patients presented a statistically significant reduction in the proportion of HLA-DR$^-$ intermediate monocytes, from 18.29% to 8.10% ($\pm$1.20%), $P \leq .004$.

On the other hand, the proportion of classical monocytes HLA-DR$^+$g was increased after chemotherapy from 77.66% ($\pm$3.88%) to 88.35% ($\pm$3.94%), $P \leq .011$, while in control individuals, the proportion of classical monocytes was 77.64%, similar to values found in patients before chemotherapy (Table 3 and Figure 4). In addition, the proportion of intermediate monocytes HLA-DR$^+$g was also increased in patients after chemotherapy compared to patients before chemotherapy, from 81.34% ($\pm$3.14%) to 92.44% ($\pm$1.29%), respectively ($P \leq .004$), reaching values similar to those found in control individuals (90.16% $\pm$ 3.30%). We found that although HLA-DR$^+$ monocytes are increased after chemotherapy, there are no differences regarding their HLA-DR expression pattern before and after chemotherapy, since we did not find differences in the percentage of HLA-DR$^+$ and HLA-DR^{++} monocytes.

The following molecules were analyzed in the plasma of the study participants: G-CSF, GM-CSF, INF-γ, interleukin (IL)-1β, IL-2, IL-4 to IL-8, IL-12 (p70), IL-13, IL-17, MCP-1, MIP-1β, and TNF-α. The concentrations of these molecules were compared between the following groups: pretreatment patients, posttreatment patients, and healthy controls. Patients with pulmonary adenocarcinoma showed an increase in the plasma concentration of the chemokine MIP-1β compared to the control group, with values of 76.87 $\pm$ 12.91 pg/mL and 35.42 $\pm$ 5.12 pg/mL, respectively, representing a 2.24-fold increase ($P \leq .02$). It is worth noting that the concentration of MIP-1β decreased to 23.06 $\pm$ 4.44 pg/mL after chemotherapy (approaching the value observed in the control individuals, $P \leq .005$). Other cytokines were also higher in the patient's plasma after chemotherapy, compared to pretreatment values. Interleukin-1β increased from 0.17 $\pm$ 0.08 pg/mL to 0.85 $\pm$ 0.33 pg/mL ($P \leq .042$), IL-5 increased from 0.63 $\pm$ 0.07 pg/mL to 2.19 $\pm$ 0.36 pg/mL ($P \leq .017$), and IL-12 increased from 2.02 pg/mL $\pm$ 0.71 to 12.59 $\pm$ 2.4 pg/mL ($P \leq .011$; Figure 5).

Discussion

Evidence accumulated for more than 20 years indicates that the immune system plays an important role in the evolution of neoplasms.[21] Thus, it has recently been proposed that the evaluation of immune subpopulations could be useful in the clinical management of patients with cancer, as well as in the staging of the disease.[19] Only a limited number of health institutions have sufficient financial and infrastructure resources to support such studies, and thus, the inclusion of this type of parameters in the staging of the disease and in the clinical control of the patients could represent a great challenge. For this reason, we evaluated the potential utility of determination of peripheral immune subpopulations that met the following criteria: (1) they had been previously associated with the evolution of neoplasms, (2) they can be quantified with relative ease, and (3) the cost of quantifying them is relatively low.

Among the lymphoid subpopulations that suppress the immune response, the present study evaluated the classical regulatory T cells (CD4$^+$CD25$^+$FoxP3$^+$). These cells inhibit the action of effector T cells through mechanisms that include the secretion of the cytokine IL-10.[22] Although it has been widely reported that the levels of classical regulatory T cells are increased in several types of cancer such as cell hepatocarcinoma and multiple myeloma,[23,24] in our study, no significant differences were found in the frequency of classical regulatory T cells between patients and control individuals; this result coincide with a study by Kotsakis *et al* that included a group of 156 patients with advanced and untreated NSCLC and 31 healthy controls.[25] That study did not find differences in Tregs (defined as CD4$^+$, CD25$^+$, and FoxP3$^+$ T cells) until they included additional markers. Currently, there is controversy

Table 2. Changes in Lymphocyte Subpopulations Before and After Platinum-Based Chemotherapy.[a,b,c]

Lymphocytes		Healthy Group, n = 9	Before Chemotherapy, n = 9	After Chemotherapy, n = 9	*P* Value
Subpopulation					
$CD4^+$		63.34 (±3.94)	56.36 (±2.01)	58.48 (±2.38)	.401
	HLA-DR g	16.75 (±3.54)	22.18 (±3.33)	26.85 (±3.54)	.379
	$HLA\text{-}DR^+$	63.66 (±5.28)	72.71 (±3.83)	69.03 (±2.79)	.308
	$HLA\text{-}DR^{++}$	37.19 (±5.23)	27.29 (±3.82)	30.98 (±2.84)	.314
	$PD1^+$	0.04 (±0.28)	0.29 (±0.16)	2.42 (±0.67)	.007
$CD8^+$		30.47 (±2.44)	39.71 (±2.70)	36.43 (±1.82)	.782
	HLA-DR g	46.28 (±9.60)	70.45 (±5.39)	73.87 (±4.56)	.705
	$HLA\text{-}DR^+$	68.17 (±5.67)	68.70 (±3.08)	67.72 (±3.77)	.964
	$HLA\text{-}DR^{++}$	31.83 (±5.66)	32.78 (±3.09)	33.53 (±3.70)	.990
	$PD1^+$	0.06 (±0.08)	0.10 (±0.13)	0.36 (±0.44)	.767[d]
Treg[e]	$CD4^+CD25^+$ $FOXP3^+$	51.41 (±8.67)	36.56 (±8.96)	ND	.420
Treg[e]	$CD4^+CD25^{+\ +}$ $FOXP3^+$	62.10 (±8.64)	49.50 (±9.94)	ND	.466
Others parameters[e]	CD4/CD8	2.01 (±0.26)	1.38 (±0.17)	1.61 (±0.09)	.572

Abbreviation: PD1, programmed death ligand 1.
[a]Median values are shown.
[b]Values in parentheses represents standard error.
[c]*P* value calculated from Student *t* test for related samples (before vs after chemotherapy contrast).
[d]*P* value calculated from Wilcoxon.
[e]CD4/CD8 Ctrl and Treg T0 has an n = 8.
[f]Statistically significant.

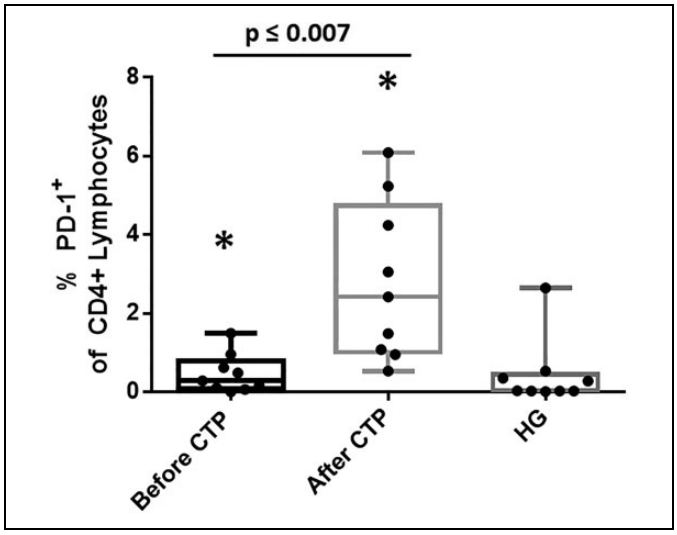

Figure 2. The frequency of $CD4^+$ $PD\text{-}1^+$ subpopulation in patients with lung adenocarcinoma increases after chemotherapy. Student *t* test for related samples with unequal variance was used for the statistical analysis. Asterisk indicates statistical significance. CTP denotes chemotherapy; HG, healthy group; PD-1, programmed death ligand 1.

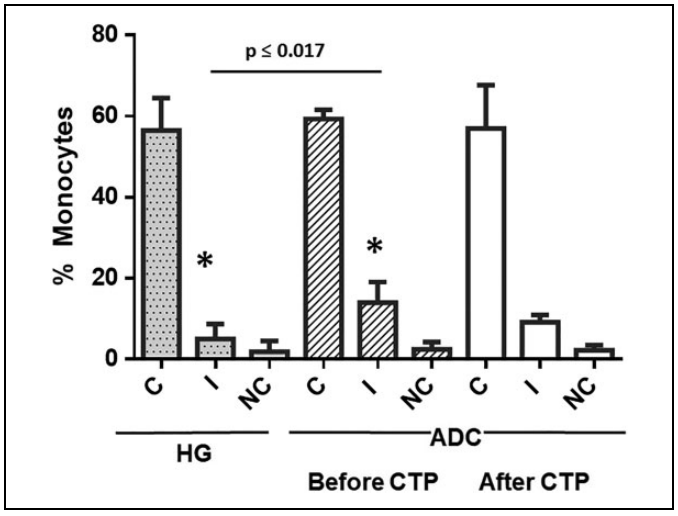

Figure 3. Increased frequency of intermediate monocytes subpopulation in patients with lung adenocarcinoma before chemotherapy compared to the healthy group. Student *t* test for nonrelated samples (HG vs ADC) and related samples (before vs after CTP) was used. Asterisk indicates statistical significance. CTP indicates chemotherapy; HG, healthy group.

about the markers that should be used to study regulatory T cells. It has been proposed that CD127, CD152, and CD45 RO should also be included to define the Treg cells, according to their differentiation and activation state. When Kotsakis *et al* used these markers, patients showed an increase in naive Treg cells defined as $CD25^{high}$, $CD127^{-/low}$, $CD152^-$, $FoxP3^{low}$, and $CD45RO^-$.[25]

Other cells that appear to play a central role in the antitumor immune response are $PD\text{-}1^+$ T lymphocytes. These cells are susceptible to inhibition by the binding of a PD-1L molecule, expressed by neoplastic cells.[26] Similar to other types of cancer, in pulmonary adenocarcinoma, the expression of PD-1L in neoplastic tissue has been associated with poor patient survival.[27] Patients showed an increase in the proportion of $CD4^+$ $PD\text{-}1^+$ T lymphocytes after chemotherapy, but not in $CD8^+$ $PD\text{-}1^+$ T lymphocytes. Since $CD4^+$ lymphocytes can collaborate with the antitumor immune response through the release of IL-2 (an important cytokine in the clonal expansion

Table 3. Changes in Monocyte Subpopulations Before and After Platinum-Based Chemotherapy.[a,b]

Monocytes		Healthy Group, n = 9	Before Chemotherapy, n =8	After Chemotherapy, n =9	*P* Value
Subpopulation					
Classical	$CD14^{++}CD16^{-}$	56.47 (± 3.52)	59.33 (± 4.22)	56.97 (± 3.32)	.85
	HLA-DR-	22.70 (± 2.95)	28.05 (±3.85)	11.38 (3.78)	.010[c]
	HLA-DR g	77.64 (± 2.98)	77.66 (± 3.88)	88.35 (± 3.94)	.011[c]
	$HLA\text{-}DR^{+}$	84.38 (±2.75)	83.60 (±2.96)	82.55 (±1.72)	.694
	$HLA\text{-}DR^{++}$	16.31 (±2.76)	16.91 (±3.0)	18.09 (±1.77)	.697
Intermediate	$CD14^{++}$ $CD16^{-}$	5.03 (± 0.98)	13.96 (± 2.31)	9.20 (± 1.02)	.065
	HLA-DR-	9.77 (± 3.23)	18.29 (± 3.13)	8.10 (± 1.20)	.004[c]
	HLA-DR g	90.16 (± 3.30)	81.34 (± 3.14)	92.44 (± 1.28)	.004[c]
	$HLA\text{-}DR^{+}$	57.62 (± 5.07)	63.94 (± 4.51)	56.27 (± 2.08)	.305
	$HLA\text{-}DR^{++}$	42.37 (± 4.98)	36.61 (± 4.46)	43.72 (± 2.14)	.302
Nonclassical	$CD14^{Neg/Low}$ $CD16^{++}$	1.90 (± 0.60)	2.50 (± 0.49)	2.25 (± 0.44)	.327[d]
	HLA-DR-	42.85 (± 9.4)	36.69 (± 11.85)	47.80 (± 10.18)	.765
	HLA-DR g	57.14 (± 9.61)	60.12 (± 11.79)	52.69 (± 10.26)	.768
	$HLA\text{-}DR^{+}$	67.85 (± 7.86)	57.23 (± 7.85)	67.17 (± 5.45)	.212
	$HLA\text{-}DR^{++}$	36.07 (± 7.50)	42.77 (± 7.85)	34.35 (± 5.43)	.224

[a]Values are median and the values in parentheses are standard error.
[b]*P* value is from Student *t* test for related samples.
[c]Statistically significant.
[d]*P* value calculated from Wilcoxon.

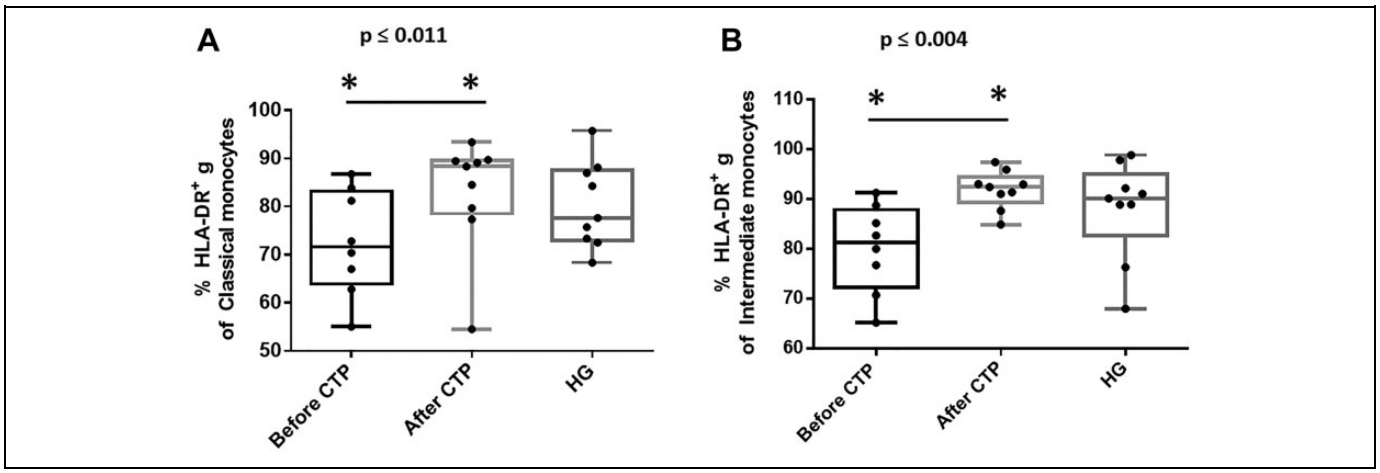

Figure 4. The frequency of $HLA\text{-}DR^{+}$ classical and intermediate monocyte subpopulations in patients with lung adenocarcinoma increases after chemotherapy and reaches levels similar to those of healthy controls. Student *t* test for related samples was used. Asterisk indicates statistical significance. CTP denotes chemotherapy; HG, healthy group.

of cytotoxic $CD8^{+}$ T lymphocytes and in the maintenance of $CD8^{+}$ memory cells), a rise in the proportion of $CD4^{+}$ $PD\text{-}1^{+}$ T cells could increase the antitumor activity of $CD8^{+}$ T lymphocytes.[28] Nevertheless, we did not find a change in IL-2 levels in the plasma of patients after chemotherapy (data not shown). We also analyzed the expression of the HLA-DR. Unlike other cell types such as monocytes, in which this molecule is constitutively expressed, HLA-DR is only expressed in activated T lymphocytes, and its expression is considered a marker of activation. Previous studies in patients with systemic lupus erythematosus found that the HLA-DR expression of $CD8^{+}$ T lymphocytes is a predictor of periods of disease activity.[29] In the present study, no differences were found in the lymphocyte expression of HLA-DR before and after chemotherapy.

The percentage of monocytes can vary in various pathological conditions: an increase in monocytes is mainly related to an active inflammatory process, which is compatible with a neoplastic process. However, it is now known that monocyte subpopulations have different properties,[30] and the balance between subpopulations of classical, intermediate, and nonclassical monocytes and their activation state could be relevant in pathological conditions. Recent studies show that classical monocytes are specialized in phagocytosis and have low proinflammatory potential. In contrast, nonclassical and intermediate monocytes have the ability to produce high quantities of cytokines. Intermediate monocytes also have a higher expression of HLA-DR and have been described as being angiogenic.[12] After activating intermediate monocytes with

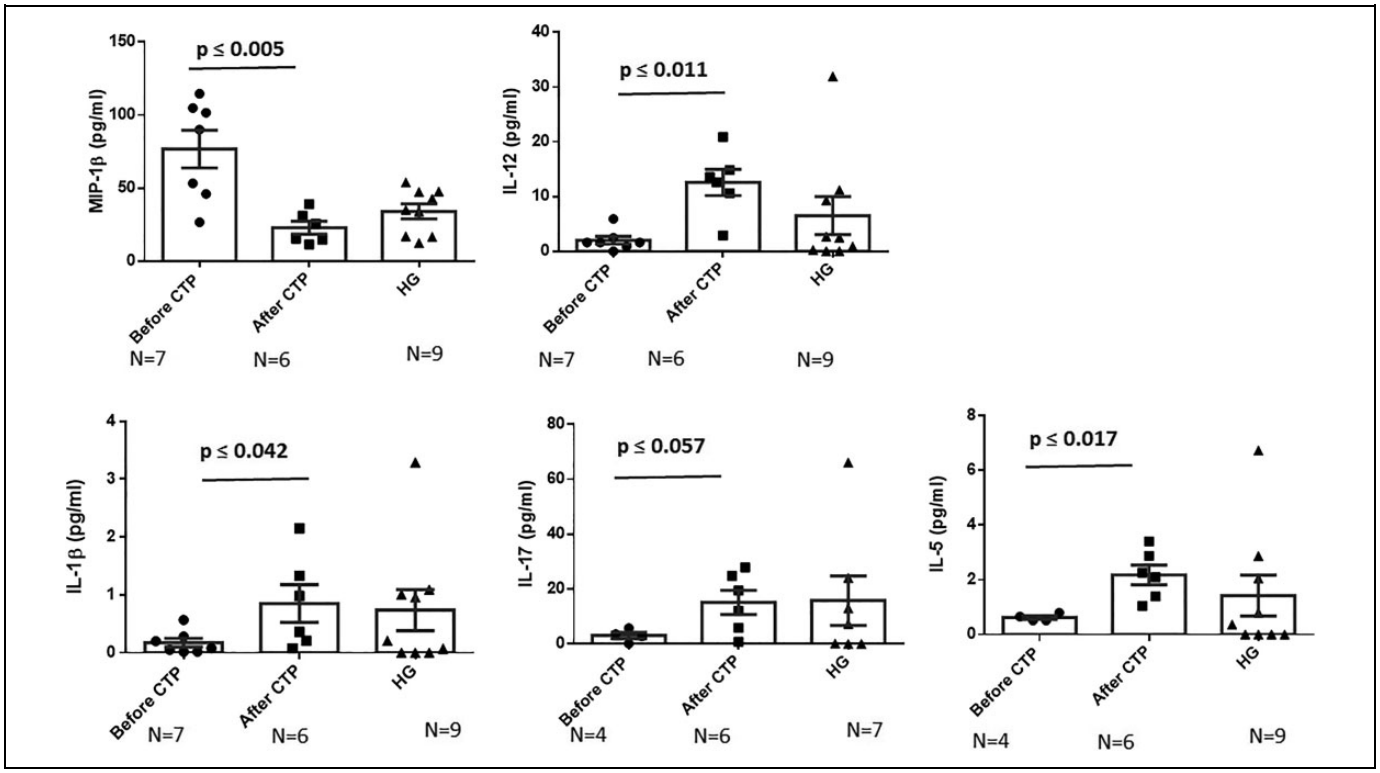

Figure 5. Plasmatic levels of IL-12, IL-1β, and IL-5 were increased in patients with lung adenocarcinoma after CTP compared to patients before treatment, whereas MIP-1 β level was decreased. Student *t* test for nonrelated samples was used, except MIP-1β and IL-5 where unequal variance was used. CTP indicates chemotherapy; HG, healthy group; IL, interleukin.|

LPS, intracellular cytometry showed that these cells produce the anti-inflammatory cytokine IL-10, while nonclassical monocytes produce the pro-inflammatory cytokines IL-1β and TNF-α.[12] We found higher levels of intermediate monocytes in patients compared to healthy individuals. After treatment, the percentage of intermediate monocytes was not significantly reduced. Since intermediate monocytes are important producers of IL-10 and are angiogenic, the increase in these cells could potentially contribute to immunosuppression and metastasis. An increase in the proportion of intermediate monocytes was previously reported in gastric cancer, in which it was associated with a higher metastatic potential.[31] We did not find significant changes in the proportion of nonclassical monocytes in postchemotherapy patients.

The expression of HLA-DR is highly variable in monocytes and its decrease has been reported in situations of systemic inflammation, sepsis, and surgical procedures, among others.[32] An increase in peripheral monocyte subpopulations ($CD14^+$, $HLA\text{-}DR^{Neg/low}$) has been previously reported in several pathological conditions, as in acute liver failure and in infections with the respiratory syncytial virus.[33,34] In the latter, the decrease in HLA-DR expression correlated with the severity of the disease.[34] It has also been reported as a marker of immunosuppression in patients with some types of cancer, such as squamous cell carcinoma of the head and neck,[16] bladder carcinoma,[35] and NSCLC.[36] Comparing different monocyte subpopulations regarding HLA-DR expression, patients showed a decrease in the proportion of classical and intermediate monocytes ($HLA\text{-}DR^+$) compared to healthy individuals. This is compatible with the reports of immunosuppression associated with the reduction of $HLA\text{-}DR^+$ expression in other neoplasms. After chemotherapy, monocyte expression of $HLA\text{-}DR^+$ showed a statistically significant increase, with values similar to those found in control individuals.

The robust monocyte expression of HLA-DR has been linked to a better prognosis in colorectal cancer,[37] while a decrease in circulating $CD14^+$ and $HLA\text{-}DR^+$ cells is associated with a poor prognosis in small cell lung cancer.[38,39] The size of the sample used in this study does not allow us to establish a correlation between an increase in the levels of classical and intermediate $HLA\text{-}DR^+$ monocytes and the survival rate of patients. However, the recovery of the monocyte expression of $HLA\text{-}DR^+$ found in patients with adenocarcinoma after chemotherapy could not be indicative of a protection in the progression of the disease since the patients died.

The observed increase in intermediate and classical $HLA\text{-}DR^+$ monocytes after chemotherapy could indicate an immunosuppression, since only phagocytic and IL-10 producing monocytes were increased, while monocytes that produce pro-inflammatory cytokines (nonclassical) did not change. Pharmacological treatment could modify the expression of HLA. Previous studies indicated that cisplatin upregulates the expression of MHC/HLA class I molecules in macrophages.[18] Additionally, the expression of HLA-DR could be affected in

patients being treated with tyrosine kinase inhibitors, because this molecule signals through tyrosine kinases.[40,41] Therefore, a compensatory mechanism could increase the expression of this molecule in the patients treated with gefitinib.

On the other hand, although the proportion of HLA-DR$^+$ in classical and intermediate monocytes varies widely in patients without treatment, it is important to emphasize that HLA-DR$^+$ monocytes showed homogeneous values after chemotherapy. This is important, since a recurrent problem in the use of cellular subpopulations as pathological prognostic indicators is the high variability between individuals. Thus, these subpopulations could be relevant in the study of the clinical evolution of patients with pulmonary adenocarcinoma.

In order to assess the inflammatory context, the concentration of a panel of molecules was determined in the plasma of patients before and after treatment, as well as in control individuals. Previous studies have described that cytokines such as IL-1β, IL-6, IL-8, and IL-17 promote the expression of transcription factors, whose target genes are involved in proneoplastic responses, such as resistance to apoptosis, cell migration, and chemoresistance[42]; other cytokines have effects on the antitumor response. In NSCLC, previous studies in the plasma of patients with squamous cell lung cancer and adenocarcinoma suggest that there are no differences in the cytokines of patients with lung cancer and control individuals.[43] Of the 17 cytokines determined in this study, the only significant differences between controls and untreated patients were in the concentration of MIP-1β, which was 2.24 (34.22-76.87 pg/mL) times higher in the plasma of the patients. It is known that this chemokine is important in the selective migration of activated CD4$^+$ T lymphocytes.[44] Therefore, an increase in the plasma concentration of MIP-1β in patients with adenocarcinoma could be part of a compensatory response to cancer-associated immunosuppression. The levels of MIP-1β decreased markedly after chemotherapy (from 76.87 to 23.06 pg/mL) and showed a tendency to approach values found in control individuals. Furthermore, the plasma concentration of IL-12 significantly increased after chemotherapy; this cytokine is known to mediate its antitumor effect through the activation of NK, CD4$^+$, and CD8$^+$ T cells.[45]

Cytokines with pro-tumoral activity (IL-1β and IL-5) showed an increase after chemotherapy, compared to the levels found in patients before treatment. Interleukin-1β is a pro-inflammatory cytokine that induces the transcription of NF-κB-driven genes, which has been correlated with a poor prognosis in breast cancer.[46] A recent report showed that the chemotherapeutic action of gemcitabine could be limited by its induction of the secretion of IL-1-β by MDSCs, thus promoting the secretion of IL-17 by CD4 lymphocytes.[47] Interleukin-17 is a cytokine with potent pro-angiogenic effects,[48] which has been reported to increase in several types of cancer, including pulmonary adenocarcinoma.[49] In the present study, we did not find a statistically significant increase in IL-17 after chemotherapy, but with a larger study sample the differences could become significant. Another chemokine that was increased after chemotherapy is IL-5; it is produced mainly by CD4$^+$ and CD8$^+$ T lymphocytes and has been reported to increase the metastatic potential of various types of cancer, including colorectal carcinoma, metastatic liver cancer, and lung cancer[50]; in the latter, IL-5 is also important for the survival of tumor cells.[50] The changes found in the plasma concentration of IL-1β and IL-5 before and after chemotherapy could be explained in part by the side effects of chemotherapy and by the time of evolution of the disease.

Conclusion

Before chemotherapy, patients with lung adenocarcinoma have lower levels of classical and intermediate HLA-DR$^+$ monocytes, compared to those present in healthy individuals. After chemotherapy, the percentage of classical and intermediate monocytes increased, approaching the values found in individuals without neoplasia, whereas nonclassical (pro-inflammatory) monocytes were not modified.

The observed increase in intermediate and classical HLA-DR$^+$ monocytes after chemotherapy could indicate an immunosuppression in advanced lung adenocarcinoma, since only phagocytic- and IL-10-producing monocytes were increased, while monocytes producing pro-inflammatory cytokines did not change; and this change was accompanied by an increase in peripheral CD4$^+$ PD-1$^+$ T cells and low patient survival.

In the peripheral inflammatory context, an increase in IL-5 was found, which could potentially favor the migration of CD4$^+$ PD-L1$^+$ T cells to tissue and thus favor immunosuppression. In the postchemotherapy group, the cytokines IL-1β and IL-12 were also increased, while MIP-1β was decreased.

Authors' Note

S. R. F. contributed to the experimental and data analysis design, procedures, manuscript design, and writing. A. H. contributed to the clinical and experimental data statistic (design and analysis). A. G. T., Y. C. C., and N. I. F. contributed to the performance sample processing, FlowJo analysis, and collection of clinical information. T. A. P., R. B. S. contributed to diagnostic and following of patients enrolled and collected clinical information. P. G. contributed to study design, founding, supervision of procedures, and approval of the final version. S. R. F. and A. H. contributed equally to the work presented.

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Supplemental Material

Supplementary material for this article is available online.

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