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Original Research

The study of CD14^{+}HLA-DR$^{-/low}$ myeloid-drived suppressor cell (MDSC) in peripheral blood of peripheral T-cell lymphoma patients and its biological function

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Abstract: Peripheral T-cell lymphoma is the generic term of a group of heterogeneous disease in non-Hodgkin's lymphoma. To investigate the quantity of CD14^{+}HLA-DR$^{-/low}$ MDSC and T cell subsets in peripheral blood of peripheral T cell lymphoma (PTCL) patients, and explore the biological functions of CD14^{+}HLA-DR$^{-/low}$ MDSC in peripheral T-cell lymphoma. Flow cytometry was used to determine CD14^{+}HLA-DR$^{-/low}$ MDSC and T cell subsets in peripheral blood of 33 peripheral T-cell lymphoma patients and 23 healthy cases; CD3 +T cells, CD14^{+}HLA-DR$^{-/low}$ MDSC cells and HLA-DR^{+} cells were selected with magnetic activated cell sorting; Biotin-labeled micro magnetic beads (CD2 / CD3 / CD28) were used to stimulate amplification of CD3 + T cells, which were co-cultured and analyzed by flow cytometry for the effect of different concentrations of CD14^{+}HLA-DR$^{-/low}$ MDSC on the proliferative activity of autologous CD3+ T cells. 1. CD14^{+}HLA-DR$^{-/low}$ cells / CD14^{+} monocytes and CD4^{+} CD25^{+}regulatory T cells Treg /CD4^{+}Tlymphocytes were 3.28% (0.40% -17.74%) and 8.81% (0.76% -78.11 %) respectively, higher than the healthy control group with statistically significant difference (P <0.05);. 2. CD4^{+}CD25^{+} Treg /CD4^{+}Tlymphocyte and CD14^{+}HLA-DR^{-}/CD14^{+} monocytes in nucleated cells of lymphoma patients were 8.81% (0.76%-78.11%) and 3.28% (0.40% -17.74%) respectively, which was in positive correlation (correlation coefficient r = 0.510), with statistically significant difference (P = 0.002); 3. When the ratio of CD14^{+}HLA-DR$^{-/low}$ and T cells was 1: 1, the proliferation inhibition of MDSC on autologous T cell is the strongest; after adding nor-NOHA, an ARG1 inhibitor, proliferation of T cells was recovered to 75.09 ± 5.18 %, IDO inhibitor 1-MT made the proliferation rate of T cells recovered to 84.05 ± 2.93%, and the proliferation rate of T cells was recovered to 97.80 ± 2.20% after combination of the IDO inhibitor and ARG1 inhibitor. CD14^{+}HLA-DR$^{-/low}$MDSC inhibit the proliferation of T lymphocytes, which may be related ARG1 and IDO.

Key words: Myeloid-derived suppressor cells; Peripheral T-cell lymphoma; CD14^{+}HLA-DR$^{-/low}$ cells.

Introduction

Lymphoma is a kind of malignant tumor, primary in the lymph nodes or lymphatic tissue. Peripheral T-cell lymphoma is the generic term of a group of heterogeneous disease in non-Hodgkin's lymphoma, accounting for 15% -20% of non-Hodgkin's lymphoma(1), including peripheral T-cell lymphoma non-specificity (PTCL NOS), angioimmunoblastic T cell lymphoma (AITL), anaplastic large cell lymphoma (ALCL) and so on (2). German highly aggressive NHL Study Group employed CHOP regimen for PTCL. 3-year OS (overall survival) and PFS (progression-free survival) of ALK-positive ALCL were 89.8% and 75.8% respectively; 3-year OS of other types were about 40% on average (3). Refractoriness and palindromic patients were commonly treated with high-dose chemotherapy combined with hematopoietic stem cell transplant, but 3-year OS was only 37%. Therefore, there is strong need to find new treatments.

Myeloid-derived suppressor cells (MDSC) is a set of heterogeneous immature myeloid cells, which is associated with the tumor-mediated immune inhibition (4-7). Currently, most of the tumor immunotherapies were in poor efficacy, mainly on account for some tumor-related immunosuppressive cells: tumor-associated macrophages TAM, regulatory T cells Treg and MDSC, in which MDSC can induce generation of Treg (8), resulting in the main obstacle of anti-tumor immunity and immunotherapy *in vivo*. Therefore, elimination of MDSC from tumor patients will benefit cancer immunotherapy for the desired effect.

MDSC in most tumors can be divided into two subgroups: monocytic MDSC (M-MDSC) and granulocytic MDSC (G-MDSC). The MDSC immune-phenotyping in mice was relatively constant, including Gr1^{+}CD11b^{+},CD11b^{+}LyC^{+} LyG$^{-/low}$ of mice M-MDSC phenotype, and CD11b^{+}LyG^{+}LyC^{-} of G-MDSC(9). MDSC plays a role of immunosuppression by reactive oxygen species (ROS), nitric oxide synthase 2 (NOS2) and arginase 1 (ARG1) and TGF-β (10).

In recent years, increased studies shows the correlation between the ratio of lymphocytes and monocytes and tumor prognosis. As reported, the prognosis of patients with newly diagnosed Hodgkin's lymphoma (11), diffuse large B-cell lymphoma(12) and peripheral T-cell lymphoma (13) was related with the lymphocytes monocytes ratio in peripheral blood. These reports from another angle confirmed the immune status of patients with lymphoma and the tumor microenvironment were associated with the prognosis. As a result, the analysis of lymphocyte subsets, in particular Treg cells and

$CD14^+HLA\text{-}DR^{-/low}$ MDSC quantities, in peripheral blood of patients with PTCL, and determination its basic functions by *in vitro* T cell inhibition assay will be of great importance in the immune therapy for PTCL. After MDSC phenotype and function determination, the administration of ARG1 and IDO inhibitors was used for exploration of the immunosuppressive mechanism of M-MDSC in PTCL and the relationship of MDSC and clinical features and prognosis of PTCL.

Materials and Methods

Study object

33 cases of peripheral T-cell lymphoma newly treated patients from January 2010 to June 2013 in our hospital and 23 healthy volunteers were recruited for the study. Diagnostic criteria for peripheral T-cell lymphoma is referred to WHO (2008 Year) lymphoma classification criteria. 33 patients had no history of infections and autoimmune diseases. Among them, the male pathological sections were diagnosed independently by at least two experienced pathologists in our hospital. This study is approved by the Medical Ethics Committee in the hospita, and all patients signed informed consent.

Experimental equipment and reagents

FACSCalibur Flow cytometry instrument: BDCo.,Ltd. (USA). The antibodies ofCD45RO/CD45RA/CD8RO/CD8RA/CD4+CD25+/CD19+/CD3+CD56+/CD14/HLA-DR: BDCo.,Ltd. (USA). Red Blood Cell Lysis Buffer (FACS Lysing Solution, 10×): BDCo.,Ltd. (USA). Human CD14 MicroBeads: Miltenyi Co.,Ltd. (Germany). Human Anti-HLA-DR MicroBeads: Miltenyi Co.,Ltd. (Germany). Human CD3 MicroBeads: Miltenyi Co.,Ltd. (Germany). Human T Cell Activation Expansion kit: Miltenyi Co.,Ltd. (Germany). Running buffer: Miltenyi Co.,Ltd. (Germany). MS Columns: MS Columns, IDO inhibitor: I-Methyl-DL-tryptophan: SigmaCo.,Ltd. (USA). ARG1inhibitor: N-hydroxy-nor-arginine/nornoha: CalbiochemCo.,Ltd. (Germany).Fetal bovine serum: Gibco BRL Co.,Ltd. (USA). PBS buffer: Gibco BRL Co.,Ltd. (USA). Violet double resistance: Gibco BRL Co.,Ltd. (USA). RPMI1640 medium: Gibco BRL Co.,Ltd. (USA).

Specimen Collection

Cells were centrifuged at 560g for 5min for discarding supernatant, and then resuspended in complete medium(89% RPMI1640, 10% fetal bovine serum,1% Violet double resistance) . The cells at concentration of 1×10^5/ml were seeded in cell culture bottles, the medium was changed every 2 to 3 days. The cells in logarithmic growth phase were selected for the next experiment.

The testing of $CD14^+HLA\text{-}DR^{-/low}$ MDSC

Peripheral blood samples were mixed homogeneously and pipetted 50ul (100ul) to flow tube; 10ul (20ul) corresponding antibodies were added for shock and 30min-reaction in the dark; then 1ml (2ml) erythrocyte lysis buffer was added for 10min-lysis in the dark, centrifuge 1243g for 5min and then discard the supernatant; washed twice with PBS, 250ul (500ul) streaming staining buffer was used to resuspended stained cells and analyze them by flow cytometry.

Function determination of CD14+HLA-DR-/low MDSC by flow cytometry

The proliferation inhibited effect of different concentrations of CD14+HLA-DR-/low cells on autologous T cells

CFSE was diluted with DMEM medium to a 5μmol/L working solution, which was incubated with equivoluminal IL-2 and micro-beads -stimulated cells suspension of 1×10^5/ ml at 37°C for 10 min; wash twice with complete culture medium and resuspend for cell count; the cells were seeded into 6-well plates, 5×10^4 cells per well

The experiment settled the experimental group (A, B, C), healthy control group (a, b, c) and negative control group (# add none of $CD14^+HLA\text{-}DR^-$), each set in three duplications; a certain proportion of $CD14^+HLA\text{-}DR^-$ cells were added in each group for co-culture (5×10^4, 2.5×10^4, 1.25×10^4 $CD14^+HLA\text{-}DR^-$ cells for Aa, Bb, Cc groups respectively) at 37°C for 96 hour in a 5% CO_2 incubator. Then centrifuge at 560g at 4°C for 10min; the supernatant was collected and stored at -80°C; PBS was used to resuspended cells after twice wash and then test by flow cytometry.

The recovery of inhibited effect of 1-MT and nor-NOHA on autologous T cells

The procedures were as the above description. A certain portion of $CD14^+HLA\text{-}DR^-$ cells were added to the experimental group and the inhibitor group for co-culture (2.5×10^4 $CD14^+HLA\text{-}DR^-$ cells for D, F, G, H groups); E group was added 2.5×10^4 $HLA\text{-}DR^+$ cells; moreover, F, G groups were added certain 1-MT, nor-NOHA respectively, while H group added 1-MT and nor-NOHA).

Statistical analysis

The statistical analysis was conducted with SPSS 20.0 software, quantitative data was presented as mean ± standard deviation if meeting the normal distribution, if not, as median (min, max). Data were compared with Student's t-test or Kruska-Wallis H nonparametric tests (Mann-Whitney U non-parametric test). $P < 0.05$ means the difference with statistically significant.

Results

Mononuclear cells in peripheral blood of PTCL patients

In order to investigate the distribution mononuclear cells in peripheral blood of PTCL patients, the peripheral blood of 33 patients with PTCL and 23 healthy volunteers were detected by flow cytometry.

As shown in Fig.1, $CD14^+$ cells / nucleated cells in peripheral blood of PTCL was significantly increased; CD3+ cells in nucleated cells were normal; the number of nucleated $CD19^+$ B cells had no change; the ratio of nucleated $CD16^+$ $CD56^+$NK cells in peripheral blood of patients with PTCL was not increased. Above results were not statistically different.

The distribution of T cell subsets of peripheral blood lymphocytes of PTCL patients demonstrated the ratio of $CD4^+CD45RO^+/CD4^+$ of patients lymphocytes

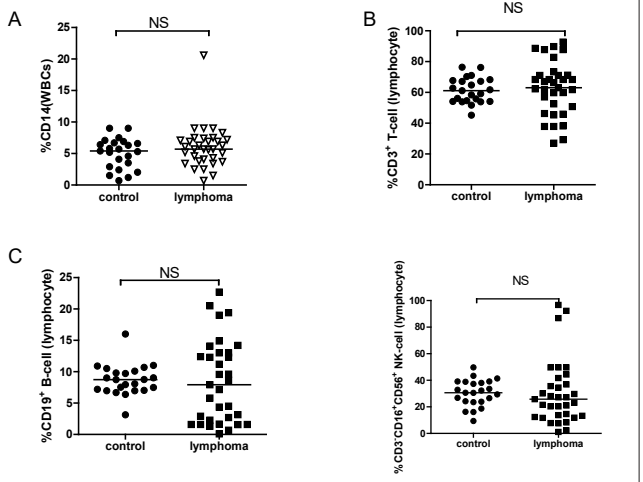

Figure 1. The distribution of mononuclear cells in peripheral blood of PTCL patients. (A) The proportion of $CD14^+$ cells/ nucleated cells in peripheral blood of peripheral T-cell lymphoma patients; (B) The proportion of CD^{3+} T cells / lymphocytes; (C) The proportion of $CD19^+$ B cells / lymphocytes; (D) The proportion of $CD^{16+}CD^{56+}$NK cells / lymphocyte ratio. (Note: NS indicates P> 0.05).

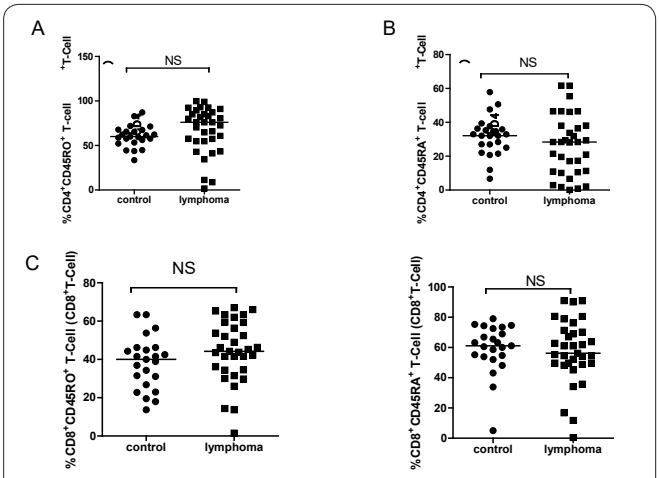

Figure 2. The distribution of mononuclear cells in peripheral blood of PTCL patients. (A) The proportion of $CD4^+CD45RO^+$ cells/ CD 4^+ cells in peripheral blood of PTCL patients; (B) The proportion of $CD4^+CD45RA^+$cells/ CD 4^+ cells; (C) The proportion of $CD8^+CD45RO^+$cells/ $CD8^+$ cells; (D) The proportion of $CD8^+$ $CD45RA^+$cells/ $CD8^+$ cells. (Note: NS indicates P> 0.05).

was 60% (33.62% -87.23%) of median , compared with the ratio of the healthy control group of 76.08% (1.65% -99.80%) of median, (T = 270.00, P = 0.068), as shown in Figure 2A. The ratio of $CD4^+CD45RA^+$cells / $CD4^+$cell was 32.12% (6.67% -57.84%) of median, compared with healthy control group of 28.37% (0.09% -61.73%), (T = 463.00, P = 0.164) of median in Figure 2B. Figure 2C illustrated the ratio of $CD8^+CD45RO^+$ cells/$CD8^+$cells was 44.20% of the median, the control group was 40.00% of meidian, (T=281.00, P=0.101). In Figure 2D, the ratio of $CD8^+CD45RA^+$cells /$CD8^+$cells was 61.15% of the median, the healthy control group was 56.20% of median(T=406.00, P=0.659). There was no statistical difference between the above results.

The proportion of $CD14^+HLA\text{-}DR^{-/low}$ cells of nucleated cells in PTCL peripheral blood increased

As illustrated in Figure 3, the ratio of $CD14^+$HLA-$DR^{-/low}$ / $CD14^+$PTCL cells in PTCL patients was 3.28% (0.40% -17.74%) , while the control group was 1.84% (0.40% -6.15%), the difference between the two groups was statistically significant (T = 208.00, P = 0.004).

The proportion of $CD4^+$ $CD25^+$ /$CD4^+$cells of nucleated cells in PTCL peripheral blood

The proportion of CD4+ CD25+ /CD4+cells of nucleated cells in lymphoma patients (median of 8.81%, 0.76% - 78.11%) showed a statistically significant difference with the control group (median of 1.64%, 0.42% - 2.83%) , (T = 80.00, P <0.001) (Figure 4).

The correlation of the proportion of $CD4^+CD25^+$/ $CD4^+$cells and $CD14^+HLA\text{-}DR^{-/low}$ / $CD14^+$ cells in peripheral blood of PTCL patients

The peripheral blood samples of 33 cases of peripheral T cell lymphoma were analyzed and it was found that the proportion of $CD4^+CD25^+$/$CD4^+$cells in the nucleated cells of lymphoma patients (median of 8.81%, 0.4% ~ 78.11%) was positively correlated (r = 0.510) with that of $CD14^+HLA\text{-}DR^{-/low}$ / $CD14^+$ cells (median of 3.28%, 0.40% ~ 17.74%), which had statistically significant difference (P = 0.002).

The function of $CD14^+HLA\text{-}DR^{-/low}$ cells

The anti-proliferation effect of different concentrations of $CD14^+HLA\text{-}DR^{-/low}$cells on autologous T cells

In order to explore the anti-proliferation effect of different concentrations of $CD14^+HLA\text{-}DR^-$ cells on autologous T cells (patients A, B, C and healthy subjects a, b, c) inhibition of proliferation activity, different concentrations of $CD14^+HLA\text{-}DR^{-/low}$ cells were selected to co-cultured with CD3+ cells labeled with CFSE

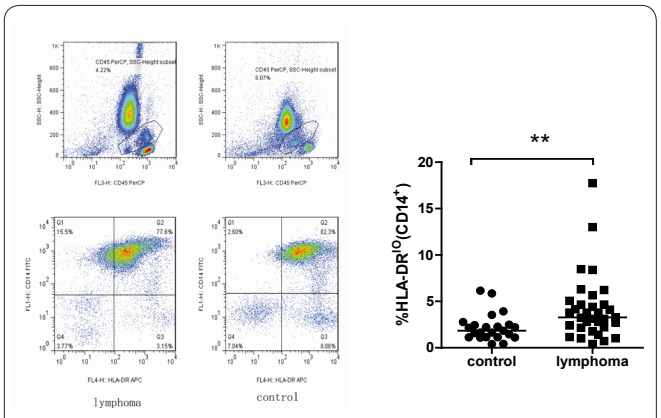

Figure 3. The ratio of $CD14^+HLA\text{-}DR^{-/low}$ / $CD14^+$PTCL cells in peripheral blood of PTCL patients. (Note: * indicates P <0.01).

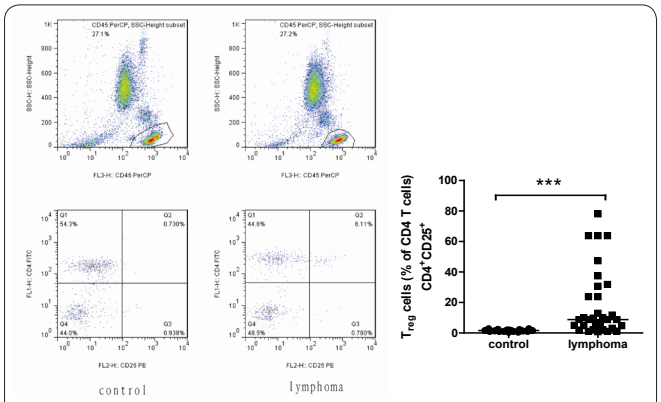

Figure 4. The proportion of $CD4^+$ $CD25^+$ /$CD4^+$cells in peripheral blood of PTCL patients. (Note: *** indicates P <0.001).

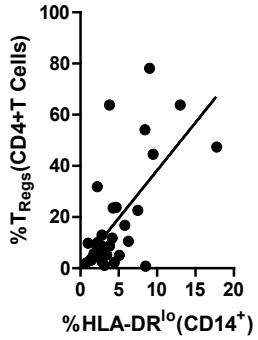

Figure 5. The correlation of $CD4^+CD25^+/CD4^+$cells and $CD14^+HLA\text{-}DR^{-/low}$ / $CD14^+$cells in peripheral blood of PTCL patients.

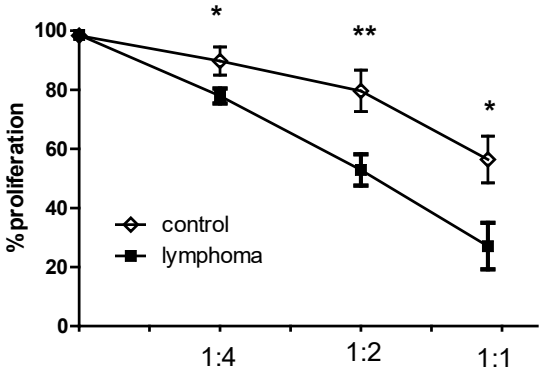

Figure 6. The anti-proliferation curve of different concentrations of $CD14^+HLA\text{-}DR^{-/low}$ cells on autologous T cells. (Note: * indicates P <0.01, ** indicates P<0.01).

working fluid. Then the following results were obtained (the proliferation rate of single CD3+ was set as 100%):

As shown in Figure 6, the proliferation rates of autologous T cells (patients and healthy subjects) co-cultured with $CD14^+HLA\text{-}DR^{-/low}$ cells were (77.90 ± 2.54)%, (52.85 ± 5.30)%, (27.11±7.86) %, (89.74 ± 4.76)%, (79.64 ± 7.04)%, (56.38 ± 7.90)% respectively. Each set of data were conducted the test of normality and homogeneity of variance, once meeting normal distribution and homogeneity of variance, T-test was carried out. The results were presented in Table 1. When the ratio of MDSC and T cells was 1: 1, the anti-proliferation effect of MDSC on autologous T cells reached the strongest.

The inhibition recovery of nor-NOHA and 1-MT on autologous T cells

The ratio of MDSC and T cells of 1:2 was selected for follow-up experiments.

IDO inhibitor 1-MT and ARG1 inhibitor nor-NOHA had significant blocking effect on the suppression of MDSC cells. In order to verify the proliferation activity recovery of autologous T cells (patients and healthy subjects) caused by IDO inhibitors and ARG1 inhibitors on MDSC cells, we chose different inhibitors acting on co-culture system of MDSC and CD3+ cells, the following results were obtained (the proliferation rate of single CD3+ was set as 100%).

nor-NOHA and 1-MT promote the secretion of IFN -γ

CD3+ cells were co-cultured with MDSC, which were added IDO inhibitor 1-MT or ARG1 inhibitor nor-NOHA respectively, or 1-MT and nor-NOHA simultaneously. The culture supernatant was separated for content detection of interferon γ by ELISA. The result indicated that addition of 1-MT or nor-NOHA promoted the secretion of interferon–γ, especially the combination addition made the highest content (379 ± 101) pg/ml, there was a significant difference between the groups (Table 3 and Figure 8).

Discussion

MDSC were derived from myeloid progenitor cells, which can be increased by tumor or inflammation. Studies have shown that the expression of MDSCs in B-cell lymphoma and Hodgkin's lymphoma is associated with poor prognosis(14), and the mechanism remains

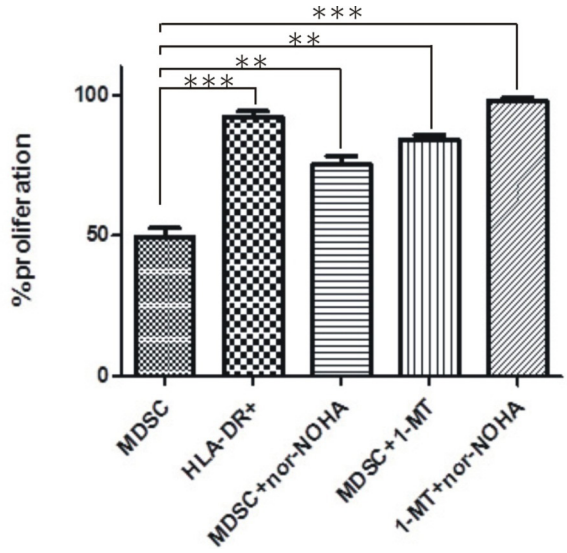

Figure 7. The proliferation recovery of different inhibitors on autologous T cell inhibited by MDSC. (Note: *** indicates P <0.001).

Table 1. The anti-proliferation effect of different concentrations of $CD14^+HLA\text{-}DR^{-/low}$ cells on autologous T cells.

	CD3+cell	M-MDSC	Proliferation rate (%)	T	P
PTCL	5×10^4	1.25×10^4	77.90±2.54	3.796	0.019
CON	5×10^4	1.25×10^4	89.74±4.76		
PTCL	5×10^4	2.5×10^4	52.85±5.30	5.268	0.006
CON	5×10^4	2.5×10^4	79.64±7.04		
PTCL	5×10^4	5×10^4	27.11±7.86	4.548	0.010
CON	5×10^4	5×10^4	56.38±7.90		

Note: PTCL: peripheral T-cell lymphoma patients; CON: healthy subjects; T: statistics follows a normal distribution and homogeneity of variance.

Table 2. The proliferation recovery of nor-NOHA and 1-MT on autologous T cells inhibited by MDSC.

	D	E	F[a]	G[b]	H[c]
CD3+cells	5×10^4	5×10^4	5×10^4	5×10^4	5×10^4
MDSC	2.5×10^4	#	2.5×10^4	2.5×10^4	2.5×10^4
proliferation rate %	49.62±5.18	92.07±3.46	75.09±5.18	84.05±2.93	97.80±2.20

Note: # Represents 2.5×10^4 HLA-DR+ cells; a represents addition of the ARG1 inhibitor nor-NOHA; b represents addition of the IDO inhibitor 1-MT; c represents simultaneously addition of nor-NOHA and 1-MT.

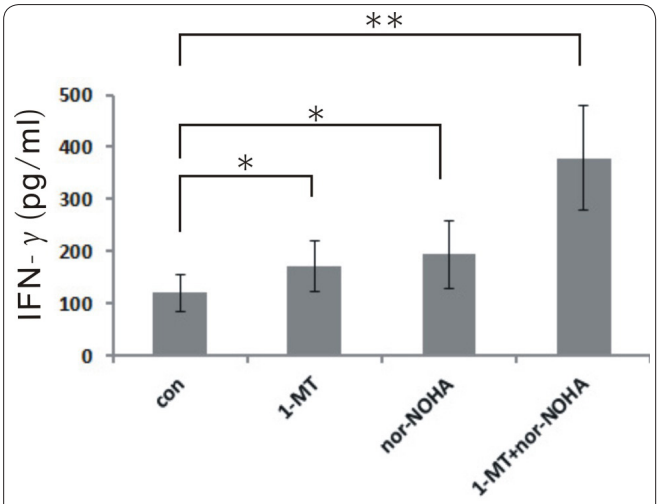

Figure 8. The effect of 1-MT and nor-NOHA on secretion of IFN-γ. (Note: * indicates P<0.05, ** indicates P<0.01).

unclear, suggesting that MDSCs may be associated with poor prognosis of PTCL. This experiment confirmed that the numbers of MDSC and Treg in peripheral T-cell lymphoma were increased, and in positive correlation. But the relationship of Treg cell amplification and MDSC is still in further study. Furthermore, MDSC in peripheral T-cell lymphoma may be partly mediated by ARG1 and IDO.

The study of Yi Lin (15)showed that the expression of $CD14^+HLA\text{-}DR^{-/LOW}$ inperipheral blood of NHL patients was reduced, and its number was related to the malignancy degree of lymphoma. As a common hematologic malignancy, the MDSC phenotype in peripheral blood of patients with chronic lymphocytic leukemia (CLL) is the MDSC of $CD14^+HLA\text{-}DR^{-/LOW}$, and CLL cells can also induce normal human monocytes into MDSC with high-expressed IDO(16). James Favaloro confirmed MDSC played an important role in the immune suppression of multiple myeloma(17). Therefore, this study analyzed the phenotype of mononuclear cell in peripheral blood by flow cytometry to reveal that M-MDSC with $CD14^+HLA\text{-}DR^{-/LOW}$ phenotype increased, which was consistent with Yi Lin's research. However, Treg cells in peripheral blood were increased, positively correlated with the number of MDSC. Treg cells and mast cells are associated with immune suppression of B-cell lymphoma, and IL-9 may be the key factor for regulating Treg cells and mast cells. After removal of Treg cells or addition of the anti-CTLA-4 antibody, the function of T cells was recovered, confirming the immunosuppression role of Treg cells in non-Hodgkin's lymphoma (18). Meanwhile, MDSC can induce the amplification of Treg cells(19).Eva Schlecker (20) found that M-MDSC in tumor-bearing mice can promote the secretion of chemokine CCR5 ligand, while Treg highly expressed CCR5. As a result, Treg cells can be recruited into the tumor by MDSC to exert immunosuppressive effect.

Table 3. The effect of 1-MT and nor-NOHA on secretion of IFN-γ.

	CON	1-MT	Nor-NOHA	CO
CD3+ cell	5×10^4	5×10^4	5×10^4	5×10^4
MDSC	2.5×10^4	2.5×10^4	2.5×10^4	2.5×10^4
IFN-γ (pg/ml)	120±35	171±49	194±65	379±101

In hematological malignancies, M-MDSC is the major type. Mougiakakos D have proved the patients have occurred the increased M-MDSC of $CD14^+HLA\text{-}DR^{-/low}$ and high expression of IDO after undergoing allogeneic hematopoietic stem cell transplantation regardless of the tumor type(21). However, MDSC didn't play immunosuppression role through IDO in all tumors. In the T cell proliferation assay *in vitro* by Han Zhang, after addition of inhibitors of ARG1, iNOS and ROS MDSC-mediated suppression of T cells was alleviated, and the activity of T cell was partially or fully recovered(22). When this test is MDSC T lymphocyte proliferation in vitro inhibition test, given ARG1 and IDO inhibitors, can partly restored T-cell proliferation, suggesting that MDSC in peripheral T-cell lymphoma may be partly mediated by ARG1 and IDO.

In summary,$CD14^+HLA\text{-}DR^{-/low}$MDSC cells have anti-proliferated effect on T lymphocytes, which is correlated withARG1 and IDO. Whether the increase of MDSCs and Tregs in peripheral blood of peripheral T-cell lymphoma is related to the clinical features and prognosis of peripheral T-cell lymphoma is still in need for further study. Systemic immunosuppression is composed by the immunosuppressive cytokines and growth factors. Therefore, further determination of cytokines in peripheral blood has a significant effect on understand the action of MDSCs and Tregs in systemic immunity of peripheral T-cell lymphoma.

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