doi: 10.13241/j.cnki.pmb.2020.18.003

利用流式细胞术评价长期冻存对 PBMCs 各亚型的影响 *

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摘要 目的：利用流式细胞术，检测长期冻存后 PBMCs 总数及各亚型的变化，评价 PBMCs 的长期冻存效果。**方法：**收集志愿者外周血 PBMCs，利用流式细胞术分析液氮冻存后 PBMCs 细胞总数及其亚型的变化。**结果：**长期冻存后，PBMCs 总细胞量和细胞活力无显著改变（P=0.19, P=0.32）；T 细胞、NK 细胞和 NKT 细胞比例无明显变化，但 B 细胞比例增多（P<0.01），单核细胞比例显著减少（P<0.001）；低温保存影响活化的 T 细胞和 Tregs 细胞数量（P<0.05, P<0.05），其中初始 Tregs 和记忆 Tregs 显著减少（P<0.05, P<0.01）。**结论：**PBMCs 长期冻存会影响 B 细胞，单核细胞、活化 T 细胞和 Tregs 细胞的活性。

关键词：外周血单个核细胞；低温冻存；淋巴细胞

中图分类号：R-33 R392.12 **文献标识码：**A **文章编号：**1673-6273(2020)18-3413-06

Using Flow Cytometry to Evaluate the Effects of PBMC Subtypes after Long-term Cryopreservation*

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ABSTRACT Objective: To explore the influence of long-term cryopreservation on the total number and subpopulation distribution of PBMCs by flow cytometry, in order to evaluate this storage method. **Methods:** PBMCs were isolated from the peripheral blood that collected from the volunteers. After long-term cryopreservation in liquid nitrogen, the changes of the total cell number of PBMCs and a serious of surface markers of different subpopulations were analyzed by flow cytometry. **Results:** The total cell number and survival rate of PBMCs did not significantly change after long-term cryopreservation (P=0.19, P=0.32). Similarly, there was no dramatic alteration in proportion of T cells, NK cells and NKT cells. While the proportion of B cells significantly increased (P<0.01), and the proportion of monocytes cells significantly decreased (P<0.001). Additionally, the proportions of activated T cells and Treg cells were dramatically affected by long-term cryopreservation (P<0.05, P<0.05). The naive Treg cells and memory Treg cells were significantly decreased (P<0.05, P<0.01). **Conclusion:** Long-term cryopreservation had a dramatic influence on proportion of B cells, monocytes, activated T cells and Treg cells in PBMCs.

Key words: PBMCs; Cryopreservation; Lymphocyte

Chinese Library Classification(CLC): R-33; R392.12 Document code: A

Article ID: 1673-6273(2020)18-3413-06

前言

人类外周血单个核细胞 (Peripheral blood mononuclear cells, PBMCs)是重要生物样本资源，常用于免疫疾病发病机制研究[1,2]，或用于免疫治疗效果评价[3]。外周血单个核细胞包括固有免疫细胞和适应性免疫细胞，固有免疫细胞可以分泌细胞因子，快速识别受体发挥免疫效应，而适应性免疫细胞可特异性识别抗原，同时产生记忆细胞发挥免疫功能。低温保存可以维持 PBMCs 的免疫活性，为免疫疾病研究提供大量样本，减少多次重复试验误差[4,5]。评价长期冻存对 PBMCs 的影响，可为免疫疾病研究中临床样本的使用提供重要参考依据。

分子标志物可用来区分 PBMCs 亚型[6]，CD 分子将 PBMCs 分为 T、B、NK 和单核细胞[7,8]，CD45RA 与 CCR7 代表 T 细胞不同分化阶段[9]，CD38 和 HLA-DR 与细胞活化相关[10-12]，CD25 和 CD127 是 Tregs 标志分子[13,14]，Tfh 高表达 CXCR5，协同 B 细胞参与体液免疫[15,16]。虽然短期冻存对这些标志物无显著影响[17,18]，但某些功能性 T 细胞如 Tregs 抑制功能降低[19]。另一方面，免疫学研究是一个长期复杂的过程，需要大量样本支持。因此，探讨 PBMCs 长期冻存效果，对免疫学研究有重要意义。

流式细胞术是 PBMCs 细胞亚群检测的标准方法[20-25]。为了明确长期冻存对 PBMC 活力及各亚型表面标志物的影响，本研究利用流式细胞术，分析液氮冷冻保存后 PBMCs 细胞总数，及其细胞亚型的变化，为 PBMCs 生物样本储存提供重要依据。

* 基金项目：国家自然科学基金项目（81770569）；中国博士后科学基金项目（2018M643871）；国家重大新药创制项目（2014ZX09508002-001）

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（收稿日期：2020-03-03 接受日期：2020-03-28）

1 材料与方法

1.1 实验器材

流式细胞仪(BD FACS Canto II Flow Cytometer,美国);超净工作台(Thermo,美国);高速离心机(Eppendorf,美国);细胞计数仪(Bio-Rad,美国)。

1.2 实验试剂

人淋巴细胞分离液(Sigma);RPIM1640(Gibco);DMSO(Sigma);PBS(Sigma);流式抗体来自 Biolegend 和 BD,具体抗体信息见表 1。

1.3 方法

1.3.1 PBMC 的收集与冻存　抽取志愿者静脉血，肝素抗凝。RPIM 1640 等体积稀释,Ficoll-hypaque 密度梯度离心(1600 rpm,25℃,30 min)，吸取中间以 PBMCs 为主的白色云雾状窄层,加入 RPIM 1640 充分洗涤两次(1300 rpm,10 min),配制 90%自体血清 +10%二甲基亚枫冻存液冻存细胞,-80℃冰箱程序降温过夜后转入液氮中长期保存。

1.3.2 PBMC 的解冻　从液氮中取出的 PBMCs 在 37℃水浴锅中快速解冻，用含有 10 %FBS 的 RPIM 1640 洗涤离心(1300 rpm,10 min)。

1.3.3 细胞染色　100 μL 细胞悬液中分别加入标记不同淋巴细胞亚型的抗体,轻微振荡混匀后避光保存 20 分钟,随后加入 400 μLPBS 混匀后上机检测,配色方案见表 1。

表 1 抗体信息

Table 1 Antibody information

	Ab	Fluorochrome	Clone	Manufacturer
T,B,Monocyte,NK,NKT	CD45	PE	HI30	Biolegend
	CD3	Percp	UCHT1	Biolegend
	CD19	APC	HIB19	Biolegend
	CD56	APC-CY7	HCD56	Biolegend
	CD16	PE-CY7	3G8	Biolegend
	CD14	FITC	M5F2	Biolegend
	Zombie	BV510		Biolegend
T subtype	CD3	PE-CY7	UCHT1	Biolegend
	CD4	FITC	RPA-T4	BD
	CD8	APC	RPA-T8	Biolegend
	CD45RA	Percp	HI100	Biolegend
	CCR7	BV510	3D12	Biolegend
	CD38	BV421	HIT2	Biolegend
	HLA-DR	APC-CY7	L243	Biolegend
Tregs,Tfh	CD3	Percp	UCHT1	Biolegend
	CD4	FITC	RPA-T4	BD
	CD25	PE	BC96	BD
	CD127	AF647	HIL-7R-M21	BD
	CD45RO	PE-CY7	UCHL1	Biolegend
	CXCR5	BV510	J252D4	Biolegend

1.3.4 流式检测　利用 BD FACSCanto II 流式细胞仪和 BD FACSDiva 软件进行流式检测，合理设门并获取 >5× 10^4 细胞数进行采集分析。细胞分群设门策略见图 1。

1.4 统计分析

利用 GraphPad prism8.0 进行统计分析，统计差异通过 Kruskal-Wallis 检验计算,P<0.05 认为具有统计学意义。流式画图软件为 FCS Express 7 Research Edition(De Novo Software)。

2 结果

2.1 长期冻存对 PBMC 总数及其活力的影响

如图 2 所示，冻存半年的 PBMCs 的细胞总数与新鲜分离的 PBMCs 总数无差异，死细胞数无差异，说明长期冻存对 PBMCs 细胞总数和细胞活力没有显著影响。

2.2 长期冻存对 PBMC 细胞表型的影响

如图 3 所示,与新鲜冻存的 PBMCs 相比,长期冻存后 B细胞比例增多(9.87 %±3.04 %),单核细胞比例显著降低(8.50 %±2.78 %),白细胞、T 细胞、NK 细胞和 NK T 细胞无统计学差异。

2.3 长期冻存对不同活化阶段 T 细胞的影响

如表 2 所示,长期冻存对 T 细胞的初始 T 细胞、效应 T 细胞和效应记忆 T 细胞都没有显著影响,但是 $CD4^+$ 的中央记忆 T 细胞对低温冻存较敏感。冻存后 CD38 和 HLA-DR 的表达量降低,活化的 CD4T 和 CD8T 显著减少。

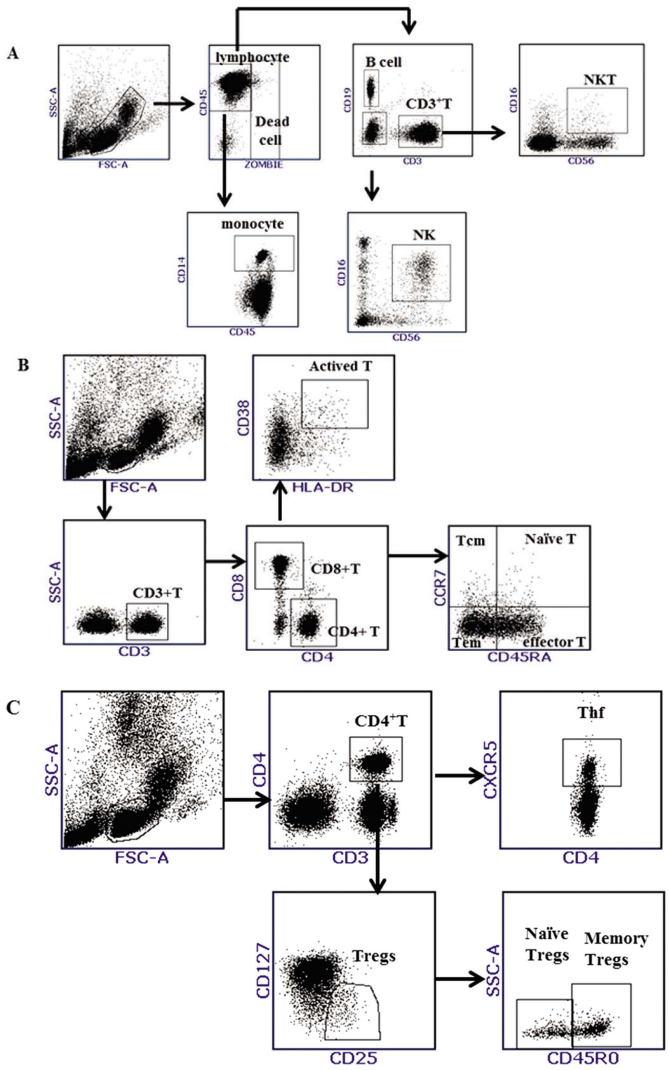

图 1 细胞分群设门流程图

Fig.1 Gating strategies for cell subtypes

Note: A, Gating strategies for PBMCs subtypes B, Gating strategies for T cell subtypes C, Gating strategies for functional T cell .

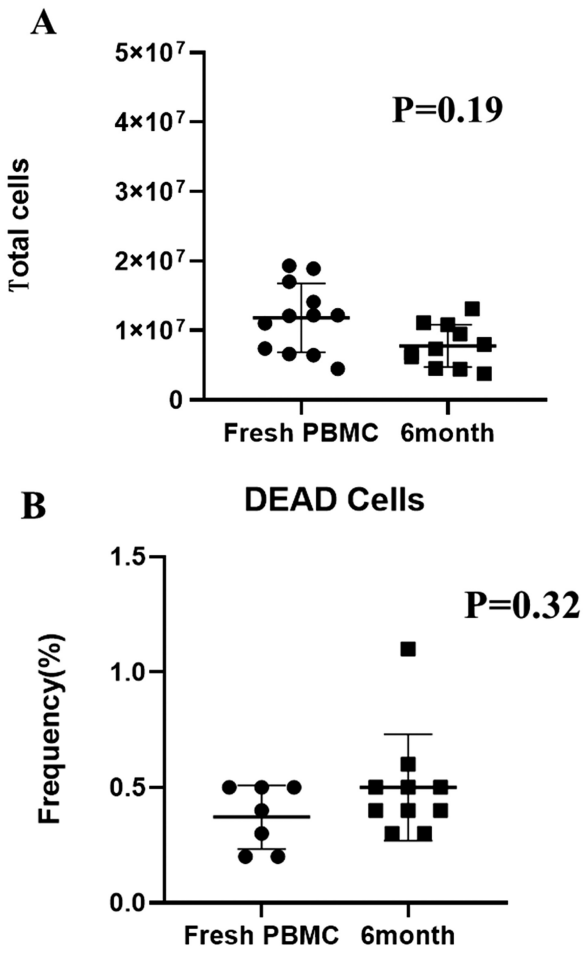

图 2 长期冻存对 PBMC 的影响

Fig.2 Effects of long-term cryopreservation on PBMCs

Note: A, total cells of PBMCs B, proportion of dead PBMCs.

2.4 长期冻存对不同功能 T 细胞的影响

如图 4 所示，CD4 T 细胞，CD8 T 细胞和 Tfh 细胞不受冻存的影响（P=0.16, P=0.07, P=0.92），如表 3 所示，Tregs 细胞、naïve Tregs 和 memory Tregs 冻存后显著减少。

3 讨论

PBMCs 是机体免疫的重要组成部分，为了获得大量细胞样本进行免疫学研究，外周血 PBMCs 收集后需低温保存[26]，但是 PBMCs 的分离技术和冷冻保存的时间都可能影响细胞数量和淋巴细胞亚群的比例[27,28]。低温保存是一种相对暴力的过程，冷冻和解冻过程都可能会引起细胞的物理和化学应激，造成细胞损伤，引起表面标志物的变化[29]。本实验在细胞冻存时采用自体血清冻存，细胞解冻 37℃培养 1h 后进行相关标志检测[30]，目的就是为了缓解这种损伤。

本研究结果显示 PBMCs 经长期冻存，细胞总数和活力与新鲜分离样本无显著差异，与佑安医院的报导一致，其研究证实 2006 年至 2015 年冻存的 PBMCs 复苏后仍具有较高活力 [31]。上述报导研究时限虽长，却并未进一步探讨低温冻存对 PBMCs 细胞亚型的影响。Jennifer 等研究结果表明短期冻存不影响 PBMCs 中免疫细胞 T、B、NK 和单核细胞的免疫学特性 [32]，然而本研究发现，长期冻存后 T、NK 和 NKT 细胞无变化，但 B 淋巴细胞增多，单核细胞显著减少，这提示 B 细胞和单核细胞对低温较敏感，长期低温刺激会影响表面标志物 CD19 和 CD14 的表达。此外，还有报道证实冻存不影响 PBMCs 中淋巴细胞亚型[33]，但会损伤初始 T 细胞、中央记忆 T 细胞、效应记忆 T 细胞和 Tregs 细胞[34,35]。本研究结果显示冻存虽不影响 T 细胞亚型，却会影响 T 细胞的活化和 Tregs 的抑制功能。活化的T 细胞与 Tregs 细胞是免疫疾病发生发展的重要影响因素，效应 T 细胞与 Tregs 细胞比例失衡会引起机体免疫调控异常[36,37]，在选择实验样本时需考虑冻存对细胞的影响。

综上所述，长期冻存的 PBMCs 可作为免疫疾病研究的生物样本资源，用于检测淋巴细胞细胞数量和 T 细胞亚型的动态变化，但不利于 T 细胞功能，如 T 细胞活化或 Tregs 抑制功能的研究。为了进一步验证长期冻存对 PBMCs 的影响，仍需对 PBMCs 细胞因子分泌能力、NK 细胞杀伤能力等细胞功能进行深入研究。

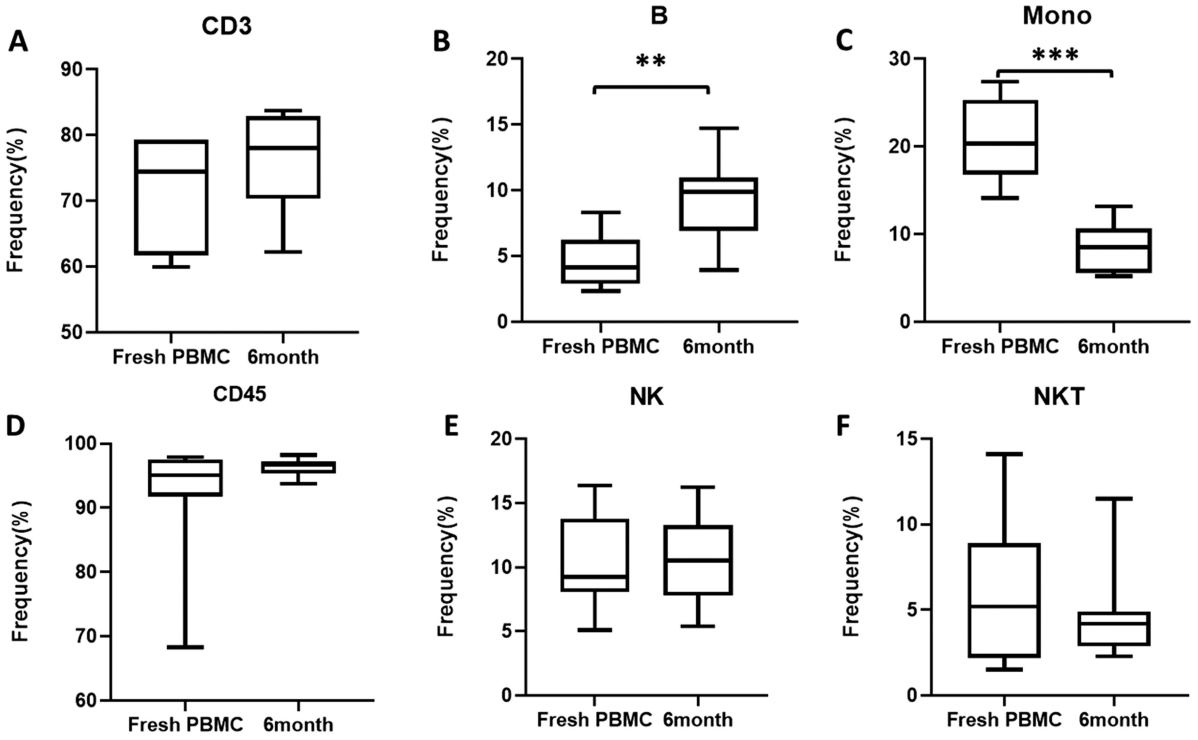

图 3 长期冻存对 PBMC 细胞表型的影响

Fig.3 Effects of long-term cryopreservation on PBMCs subtypes

Note: A, T cell gate B, B cell gate C, monocyte gate D, lymphocyte gate E, NK cell gate F, NKT cell gate. $^{**}P<0.01$,$^{***}P<0.001$.

表 2 长期冻存对淋巴细胞中不同活化阶段 T 细胞的影响(%, mean±SD)

Table 2 Effects of long-term cryopreservation on different stage of actived T cells(%, mean±SD)

$CD3^+$Tcell			
	Fresh PBMC(%)	Cryopreserved 6month(%)	*P*
CD4			
Naïve	1.06±0.68	1.07±1.5	ns
Central memory	3.14±1.17	4.76±1.57	*
Effector	20.91±13.8	18.98±10.81	ns
Effector memory	77.17±13.7	75.68±10.87	ns
Actived	1.57±1.1	0.76±0.63	*
CD8			
Naïve	2.04±1.27	2.37±0.92	ns
Central memory	1.46±1.04	1.97±0.78	ns
Effector	40.31±16.27	55.22±9.39	ns
Effector memory	53.57±16.38	41.28±9.05	ns
Actived	2.68±2.80	0.96±0.57	*

Note: $CD3^+$T was divided into $CD4^+$ and $CD8^+$T cells, $CD4^+$T cells and $CD8^+$T cells were divided into naive $CCR7^+CD45RA^+$, central memory $CCR7^+CD45RA^-$, effector $CCR7^-CD45RA^+$ and effector memory $CCR7^-CD45RA^-$, Activated $CD4^+$T cells and $CD8^+$T cells were $CD38^+HLA\text{-}DR^+$, ns(No significant difference), $^*P<0.05$.

参 考 文 献(References)

[1] Ryo K, Makoto Y, Shinya U, et al. Increased Tim-3+ T cells in PBMCs during nivolumab therapy correlate with responses and prognosis of advanced esophageal squamous cell carcinoma patients [J]. Cancer Immunology & Immunotherapy, 2018, 67(29): 1673-1683

[2] Lauren L, Renee N D, Italia G, et al. PD-1 and PD-L1 expression on PBMC subsets in normal individuals and cancer patients [J]. Journal for Immunotherapy of Cancer, 2014, 2(Suppl 3): P152-P152

[3] Wong E C C, Maher V E, Hines K, et al. Development of a clinical-scale method for generation of dendritic cells from PBMC for use in cancer immunotherapy[J]. Cytotherapy, 2001, 3(1): 19-29

[4] Crispin J C, Hedrich C M, Suárez-Fueyo A, et al. SLE-Associated

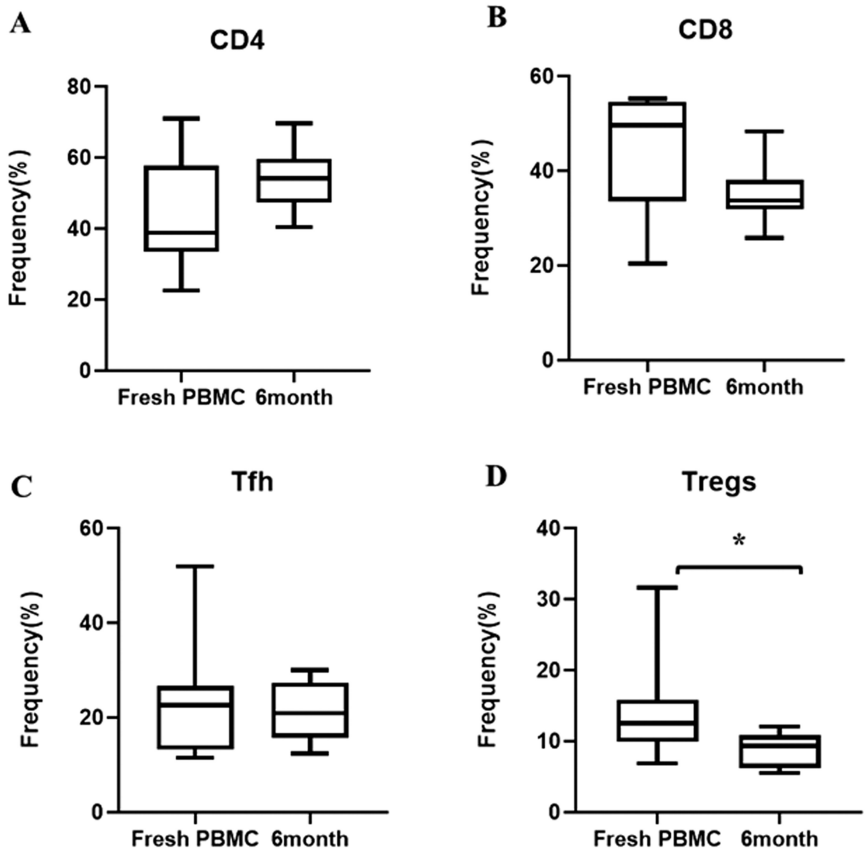

图 4 长期冻存对淋巴细胞功能 T 细胞的影响

Fig. 4 Effects of long-term cryopreservation on functional T cell

Note: A, $CD4^+$T cells B, $CD8^+$T cells C, Tfh cells D, Tregs.*P<0.05.

表 3 长期冻存对初始和记忆 Tregs 的影响(%, mean±SD)

Table 3 Effects of long-term cryopreservation on naïve Treg sand memory Tregs(%, mean±SD)

Tregs			
	Fresh PBMC(%)	Cryopreserved 6month(%)	*P*
Naïve Tregs	3.72±2.36	2.98±1.17	*
Memory Tregs	11.86±6.54	6.38±2.07	**

Note: Tregs were divided into $CD45RO^-$ naiveTregs and $CD45RO^+$ memory Tregs.*P<0.05,**P<0.01.

Defects Promote Altered T Cell Function [J]. Critical Reviews in Immunology, 2018, 37(1): 39-58

[5] Vinje O, F Rre Y, Degre M, et al. Natural Killer (NK) Cell Activity of Peripheral Blood Lymphocytes from Patients with Bechterew\"s Syndrome (Ankylosing Spondylitis) [J]. Scandinavian Journal of Rheumatology, 1984, 13(4): 297-302

[6] Cheng M, Chen Y, Xiao W, et al. NK cell-based immunotherapy for malignant diseases [J]. Cellular & Molecular Immunology, 2013, 10 (3): 230-252

[7] Sara S, De V T J, Vincent E. Monocyte Heterogeneity: Consequences for Monocyte-Derived Immune Cells [J]. Journal of Immunology Research, 2016, 1-10

[8] Hausman M S, Brosman S, Snyderman R, et al. Defective monocyte function in patients with genitourinary carcinoma [J]. Journal of the National Cancer Institute, 1975, 55(5): 1047

[9] Mao F Y, Hui K, Zhao Y L, et al. Increased tumor-infiltrating CD45RA-CCR7- regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress [J]. Cell Death & Disease, 2017, 8(8): e3002

[10] Lehmann J S, J. C J, Micheline C, et al. T cell subtypes and reciprocal inflammatory mediator expression differentiate P. falciparum memory recall responses in asymptomatic and symptomatic malaria patients in southeastern Haiti[J]. Plos One, 2017, 12(4): e174718

[11] Gonzalez S M, Taborda N A, Correa L A, et al. Particular activation phenotype of T cells expressing HLA-DR but not CD38 in GALT from HIV-controllers is associated with immune regulation and delayed progression to AIDS[J]. Immunologic Research, 2016, 64(3): 765-774

[12] Brinkmann C R, Hjen J F, Rasmussen T A. Treatment of HIV-Infected Individuals with the Histone Deacetylase Inhibitor Panobinostat Results in Increased Numbers of Regulatory T Cells and Limits Lipopolysaccharide-Induced Inflammatory Responses [J].

mSphere, 2018, 3(1): e00616-17

[13] Czarnowicki T, He H, Leonard A, et al. The Major Orphan Forms of Ichthyosis Are Characterized by Systemic T-Cell Activation and Th-17/Tc-17/Th-22/Tc-22 Polarization in Blood [J]. Journal of Investigative Dermatology, 2018, 138(10): 2157-2167

[14] Sakaguchi S, Yamaguchi T, Nomura T, et al. Regulatory T Cells and Immune Tolerance[J]. Cell, 2008, 133(5): 787

[15] Wan Z, Lin Y, Zhao Y, et al. TFH cells in bystander and cognate interactions with B cells [J]. Immunological Reviews, 2019, 288(1): 28-36

[16] Elias S, Milne K, Chui C, et al. Analysis of peripheral blood B and Tfh cells as predictors of antibody responses in individuals receiving candidate blood-stage malaria vaccines in a Phase Ia clinical trial[J]. Malaria Journal, 2014, 13(1 Supplement): P28

[17] Johannes, Trück, Ruth, et al. Effect of cryopreservation of peripheral blood mononuclear cells (PBMCs) on the variability of an antigen-specific memory B cell ELISpot [J]. Human Vaccines, 2014, 10(8): 2490-6

[18] Lemieux J, Jobin C, Simard C, et al. A global look into human T cell subsets before and after cryopreservation using multiparametric flow cytometry and two-dimensional visualization analysis [J]. Journal of Immunological Methods, 2016, 434, S1000151553

[19] Weiner J, Duran-Struuck R, Zitsman J, et al. Restimulation After Cryopreservation and Thawing Preserves the Phenotype and Function of Expanded Baboon Regulatory T Cells [J]. Transplantation Direct, 2015, 1(1): 1

[20] Fouda A, Tahsini M, Khodayarian F, et al. A Fluorescence-based Lymphocyte Assay Suitable for High-throughput Screening of Small Molecules [J]. Journal of Visualized Experiments Jove, 2017, (121): e55199

[21] Liwei G E, Wang X. Peripheral blood cell determination for acute leukemia recurrence by flow cytometry [J]. Laboratory Medicine, 2018, 33(3): 239-241

[22] Ciuceis C D, Rossini C, Airò P, et al. Relationship Between Different Subpopulations of Circulating $CD4^+$ T-lymphocytes and Microvascular Structural Alterations in Humans[J]. American Journal of Hypertension, 2017, 30(1): 51-60

[23] Chen, Liye. The role of ERAP1 and HLA-B27 in ankylosing spondylitis pathogenesis[J]. EThOS, 2013

[24] Chen B, Li J, He C, et al. Role of HLA-B27 in the pathogenesis of ankylosing spondylitis (Review) [J]. Molecular Medicine Reports, 2017, 15(4): 1943-1951

[25] Boldt A, Borte S, Fricke S, et al. Eight color immunophenotyping of T, B, and NK cell subpopulations for characterization of chronic immunodeficiencies[J]. Cytometry B Clin Cytom, 2014, 86(3): 191-206

[26] Mallone R, Mannering S I, Brooks Worrell B M, et al. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen reactive T cell responses: position statement of the T Cell Workshop Committee of the Immunology of Diabetes Society [J]. Clinical & Experimental Immunology, 2011, 163(1): 33-49

[27] Germann A, Oh Y, Schmidt T, et al. Temperature fluctuations during deep temperature cryopreservation reduce PBMC recovery, viability and T-cell function[J]. Cryobiology, 2013, 67(2): 193-200

[28] Lockmann A, Seitz C S, Schön M P, et al. Creeping eruption and eosinophilic folliculitis: Atypical cutaneous larva migrans[J]. Journal of German Society of Dermatology, 2018, 16(2): 202-204

[29] Jeremy, Anderson, Zheng, et al. Effect of peripheral blood mononuclear cell cryopreservation on innate and adaptive immune responses[J]. Journal of Immunological Methods, 2019, 465: 61-66

[30] Martha, Holland, Rachel, et al. Separation, banking, and quality control of peripheral blood mononuclear cells from whole blood of melanoma patients[J]. Cell Tissue Bank, 2018, 19(4): 783-790

[31] Jianping S, Mengdan, Kang Li, et al. Quality of Cryopreserved Peripheral Blood Mononuclear Cells Recovered from the Hepatitis/AIDS Biobank[J]. Biopreservation and biobanking, 2018, 1-5

[32] Lemieux J, Jobin C, Simard C, et al. A global look into human T cell subsets before and after cryopreservation using multiparametric flow cytometry and two-dimensional visualization analysis [J]. Journal of Immunological Methods, 2016, 434, S1000151553

[33] Tompa A, Nilsson-Bowers A, Faresjö M. Subsets of $CD4^+$, $CD8^+$, and CD25 hi Lymphocytes Are in General Not Influenced by Isolation and Long-Term Cryopreservation [J]. Journal of Immunology, 2018, 201 (6): i1701409

[34] Weinberg A, Song L Y, Wilkening C, et al. Optimization and Limitations of Use of Cryopreserved Peripheral Blood Mononuclear Cells for Functional and Phenotypic T-Cell Characterization [J]. Clinical & Vaccine Immunology Cvi, 2009, 16(8):1176-1186

[35] Sattui S, de la Flor C, Sanchez C, et al. Cryopreservation modulates the detection of regulatory T cell markers [J]. Cytometry Part B Clinical Cytometry, 2012, 82B(1): 54-58

[36] Horst A K, C W, C S, et al. Carcinoembryonic antigen-related cell adhesion molecule 1 controls IL-2-dependent regulatory T-cell induction in immune-mediated hepatitis in mice [J]. Hepatology, 2018, 68(1): 200

[37] Chen J, Hou X, Jia H, et al. Regulatory T cells with a defect in inhibition on co-stimulation deteriorated primary biliary cholangitis [J]. Oncotarget, 2017, 8(65): 108406-108417