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Functional inactivation of lymphocytes by methylene blue with visible light

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Transfusion of allogeneic white blood cells (WBCs) may cause adverse reactions in immunocompromised recipients, including transfusion-associated graft-versus-host disease (TA-GVHD) which is often fatal and incurable. In this study, the in vitro effects of methylene blue with visible light (MB+L) treatment on lymphocyte proliferation and cytokine production were measured to investigate whether MB+L can be used to prevent immune reactions resulting from transfused lymphocytes. WBCs and 3μM MB were mixed and transferred into medical PVC bags which were then exposed to visible light. Gamma irradiation was conducted as parallel positive controls. Untreated group was the cells without treatment. All groups were tested for the ability of cell proliferative and cytokine production upon stimulation. After incubation with mitogen phytohemagglutinin (PHA) or plate-bound anti-CD3 plus anti-CD28, the proliferation of MB+L/gamma-irradiation treated lymphocytes was significantly inhibited ($P<0.01$) as compared to untreated ones; Proliferation inhibitive rate of MB+L group was even higher than that of gamma irradiated cells (73.77 ±28.75% *vs* 44.72 ±38.20%). MB+L treated cells incubated up to 7days with PHA also showed no significant proliferation. The levels of TNF-α, IFN-γ, IL-6, IL-8, IL-10 and IL-1β present in the supernatant of MB+L treated lymphocytes upon stimuli were significantly lower than those of untreated lymphocytes. These results demonstrated that MB+L treatment functionally and irreversibly inactivated lymphocytes by inhibiting lymphocyte proliferation and the production of cytokines. MB+L treatment might be a promising method for prevention of adverse immune responses caused by WBCs.

1. Introduction

Functional white blood cells (WBCs) from immunocompetent donor may lead to adverse transfusion effects in recipients during allogeneic blood transfusions, including transfusion-associated graft-versus-host disease (TA-GVHD) which is uncommon but always fatal[1]. TA-GVHD results from the engraftment and proliferation of viable donor T lymphocytes which recognize the host tissues and organs as targets and destroy them. In the absence of preventive measures, incidence rate of TA-GVHD ranges from 0.1% to 1%. The disease usually develops between 1-4 weeks after transfusion with skin, gastrointestinal tract, liver or/and bone marrow damages. Due to the lack of specific clinical manifestations, relatively small number of cases and the lack of awareness of this disease by clinicians, TA-GVHD can easily be misdiagnosed. All these above lead to a high mortality rate of over 90%[2-5]. Currently there is no available treatment for this disease so that prevention is the most effective measure to reduce its occurrence.

Gamma irradiation and leukoreduction were commonly adopted to reduce the risk of TA-GVHD. Gamma irradiation is the accepted measure for TA-GVHD prevention in immunocompromised patients or patients who are susceptible to it. However, in practice, not all blood units containing WBCs are irradiated due to several factors, such as limited use of irradiators, safety of operators and irradiation damage on red cell functions. Thus the validity of this procedure is based on proper identification of patients at potential risk. Leukoreduction is capable of WBCs removal by approximately 1000-fold with a residual number fewer than 5×10^6 per unit and has been considered inadequate for risk elimination[4, 6-7].

Nucleic acid-targeted pathogen reduction technologies (PRTs) are proved to be effective in white blood cell inactivation. Among the PRTs in use or in test, methylene blue plus visible light (MB+L) has been demonstrated to mediate nucleic acid lesions and inhibit pathogen replication which has been approved for plasma inactivation in China and some European countries[8]. The effectiveness and safety of this method have been confirmed. It is well known that upon excitation with light, methylene blue (MB)

can intercalate with nucleic acids and mediate strand breaks at guanosine sites, form 8-hydroxy-2'-deoxyguanosine or generate RNA–protein cross-linkages[9-11]. Based on its photochemical mechanisms, this technology might also possess inactivation effects on lymphocytes. However, few studies have been conducted to verify this hypothesis.

The aim of the present study was to investigate the functional inactivation effect of MB+L treatment on white blood cells by measuring proliferative activity and levels of cytokines produced by lymphocytes. Meanwhile, gamma irradiation was also conducted as positive control to assess the inactivation effects of MB+L.

2. Materials and methods

Preparation of WBCs

Whole blood was collected from randomly selected healthy donors and anticoagulated with acid citrate dextrose (ACD). Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque density gradient centrifugation from buffy coat within 6h after blood collection. After overnight culture at 37℃, lymphocytes were purified and resuspended in autologous plasma with a concentration of 1×10^6cells/mL.

Lymphocyte inactivation by MB+L/gamma-irradiation treatment

The photometric data of light source used for MB+L treatment and the absorption spectrum of MB were shown in Fig 1, which indicated that the absorption of MB matches with the photometric spectrum of the light source. The total intensity of MB+L irradiation was represented by illuminance (lux).

Cell suspensions were divided into three groups, namely MB+L group, gamma-irradiation group and untreated group. Methylene blue was added into cells to form a final concentration of 3μM followed by transferring the suspension into PVC blood bags. Cells were exposed to fluorescent light (MASTER TL-D Xtra, Philips) from both sides with illuminance of 35000 lux for 50min. Gamma-irradiated cells were treated by blood irradiator (Gammacell 3000 Elan, MDS Nordion) with central dose of 25Gy. Untreated group was the cells with no MB+L treatment or gamma-irradiation.

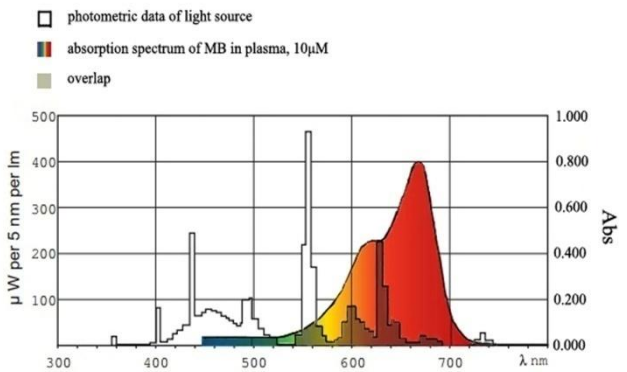

Fig.1 Photometric data of light source MASTER TL-D Xtra, the absorption spectrum of MB in plasma and the overlap of MB and the light source are shown. The absorption spectrum of 10μM MB is shown in the range between 450nm to 750nm with two peaks at 620nm and 665nm, respectively. The emission spectrum of the used light source ranges from 350nm to 740nm, which covers the absorption spectrum of MB.

After treatment, all cells were centrifuged and resuspended in RPMI Medium 1640 for culture.

Immunophenotyping

Lymphocytes were stained with a panel of antibodies (CD3/CD8/CD45/CD4 BD multitest, BD Biosciences) to define various T cell subpopulations. Cells of all groups (5×10^5cells/sample) were centrifuged at 300×g for 5 minutes. The cell pellets were washed twice and resuspended in 80μL PBS. The cells were incubated with 20μL of antibody mixtures in dark for 15min and then analyzed by using flow cytometer (FACS Calibur, BD Biosciences).

Proliferation assays

The ability of MB+L/gamma-irradiation treated and untreated lymphocytes to proliferate in response to mitogen phytohemagglutinin (PHA) or to plate-bound anti-CD3 plus anti-CD28 (anti-T-cell receptor [TCR]) antibodies were assayed.

For PHA stimulation, the final concentration of 0.5μg/mL PHA was added to cell suspensions in 24-well plates and cells were cultured for 72h before tested by Cell Proliferation ELISA kit (Roche). Only proliferating cells which incorporated BrdU into their DNA can be detected by anti-BrdU fragments through ELISA. The results were represented in O.D. at wavelength of 450 nm. Besides, the proliferative ability of treated and untreated lymphocytes incubated with PHA for 7days was also detected to demonstrate the irreversibility of the treatment.

For stimulation by a combination of TCR antibodies, 5ug/mL anti-CD3 and 2.5ug/mL anti-CD28 in PBS were plated in 24-well plates and cultured at 37℃ overnight. Cell samples of each group were labeled by CFSE and incubated for 10min at 37℃ followed by washing with RPMI 1640 medium. The antibody-bound plates were washed twice by PBS before adding labeled cells of 1×10^6 per well and culturing for 72h. Successive generations of proliferated cells were detected by flow cytometry.

Measurement of cytokine levels

To measure the ability of the untreated or treated lymphocytes to secrete cytokines, cells from the different treatment groups were stimulated with PHA or with bound anti-CD3 plus anti-CD28 for 72 hours. Supernatants were collected and stored at -20 ℃ until assayed. Levels of inflammatory cytokines (interleukin IL-1β, IL-6, tumor necrosis factor TNF-α, IL-10, IL-8, and IL-12 p70) and Th1/Th2 cytokines (interferon IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10) produced in response to stimuli were assayed using CBA assay kits (BD Biosciences) according to manufacturer's directions.

Statistical analysis

The significance of comparisons between MB+L/gamma-irradiation treated and untreated lymphocytes was analyzed using Microsoft Excel version 2007.

3.Results

Effects of MB+L/gamma-irradiation treatment on Subpopulations of lymphocytes

Analysis of lymphocytes subpopulations of MB+L/gamma-irradiation or untreated groups obtained from six different donors was performed. It was observed that there was individual variation in the proportion of $CD3^+$ cells in untreated cells from different donors (56%-81%). No statistical significant difference in immunophenotype was observed among MB+L, gamma-irradiation or untreated groups (Table 1). These results showed that MB+L treatment did not significantly alter the distribution of lymphocyte subpopulations.

Table 1. Effects of MB+L/gamma-irradiation treatment on Subpopulations of lymphocytes (n=6)

Immunophenotype	Untreated (%)	γ-irradiation (%)	MB+L (%)
CD3+	66.17±8.28	66.50±8.14	68.00±6.99
CD3+CD8+	27.67±3.01	26.50±2.88	28.00±3.41
CD3+CD4+	30.50±3.45	30.00±5.02	31.17±3.49
CD3+CD4+CD8+	0.17±0.41	0.00±0.00	0.00±0.00
CD4+/CD8+	1.11±0.21	1.16±0.24	1.13±0.17

There was no significant difference between lymphocyte subpopulations of untreated and MB+L/gamma-irradiation groups ($p>0.05$)

Proliferation inhibition of MB+L/gamma-irradiation treated lymphocytes

The proliferation ability of MB+L/gamma-irradiation treated or untreated cells in response to stimuli were tested by incubating lymphocytes with mitogen PHA or with anti-CD3 plus anti-CD28. The results demonstrated that MB+L or gamma-irradiation treatment inhibited lymphocyte proliferative response to PHA significantly ($P<0.05$) (Table 2). The inhibitive rate of proliferation was calculated according to the following formula: Inhibitive rate of proliferation (I):

$$I=\left[1-\left(O.D._{treated}^{PHA+}-O.D._{treated}^{PHA-}\right)\div\left(O.D._{untreated}^{PHA+}-O.D._{untreated}^{PHA-}\right)\right]\times 100\%$$

I of MB+L treated cells was 73.77±28.75%, higher than that of gamma-irradiated cells (I=44.72±38.20%).

For cells stimulated with anti-CD3 plus anti-CD28, it showed that, untreated cells had high proliferative capability with 3 cell division generations, while MB+L treated and gamma-irradiated cells had no cell divisions (Figure 2). The results indicated that the proliferative ability of lymphocytes was entirely suppressed by MB+L or gamma-irradiation treatment.

After 7 days of incubation with PHA stimulation, untreated and gamma-irradiated cells proliferated significantly. In contrast, MB+L treated lymphocytes showed barely any proliferation which indicated that MB+L treatment is irreversible for lymphocyte inactivation (Figure 3).

Cytokine production of MB+L/gamma-irradiation treated lymphocytes

Cytokine production of untreated or MB+L/gamma-irradiation treated lymphocytes after induction of stimuli were measured by using CBA assay kits. Incubation of cells with PHA resulted in barely detectable levels of IL-4 and IL-12p70 in the supernatants from either MB+L, gamma-irradiation treated or untreated lymphocytes. However, PHA induced high levels of IFN-γ, IL-1β, IL-6, IL-8 and IL-10 secretion in untreated cells but not in MB+L/gamma-irradiation treated cells with significant difference ($p<0.05$, Table 2).

Cells incubated with bound anti-CD3+CD28 produced low levels of IL-4 and IL-12p70 in the supernatants from all groups. Both MB+L and gamma-irradiation treatment suppressed the production of TNF-α, IFN-γ, IL-8 and IL-10 significantly, while only MB+L treatment lowered the secretion levels of IL-1β and IL-6 ($p<0.05$). These results indicated that MB+L/gamma-irradiation treated lymphocytes were inhibited from producing

Table 2. Effects of MB+L/gamma-irradiation treatment on lymphocytes proliferation upon stimulation of PHA

Sample No.	Untreated		γ-irradiation		Inhibited rate (I, %)	MB+L		Inhibited rate (I, %)
	PHA-	PHA+	PHA-	PHA+*		PHA-	PHA+*	
1	0.148	2.555	0.057	2.283	7.51	0.052	0.402	85.47
2	0.576	0.659	0.156	0.477	0.00	0.270	0.232	100.00
3	0.697	1.061	0.158	0.552	0.00	0.302	0.624	11.38
4	0.331	0.626	0.128	0.218	69.65	0.040	0.121	72.37
5	0.271	0.846	0.000	0.561	2.38	0.022	0.378	38.05
6	0.453	0.787	0.264	0.347	75.05	0.236	0.250	95.81
7	0.078	0.378	0.053	0.129	74.42	0.032	0.123	69.52
8	0.030	0.215	0.025	0.090	64.68	0.027	0.072	75.68
9	0.075	0.842	0.357	0.216	100.00	0.062	0.143	89.43
10	0.229	0.575	0.194	0.355	53.51	0.343	0.330	100.00
$\bar{X}\pm$ Std	0.289±0.226	0.854±0.645	0.139±0.112	0.523±0.640	44.72±38.20	0.139±0.132	0.268±0.170	73.77±28.75

* MB+L or gamma-irradiation treatment inhibited lymphocyte proliferative response to PHA significantly when compared to untreated lymphocytes ($P<0.01$).

cytokines. Besides MB+L treatment was more powerful in prevention of some cytokine secretions.

Discussions

Pathogenesis of TA-GVHD is that active allogeneic lymphocytes transfused into patients cannot be promptly removed but survive to proliferate and recognize the recipients' body as non-self so as to carry out immune attack on tissues and organs of recipients. In the absence of preventive measures, one case of TA-GVHD may have occurred per 400-10000 units of red blood cells transfused[14]. Early symptoms of TA-GVHD are fever, rash, diarrhea and hepatitis. Since these symptoms usually appear 1-2 weeks after transfusion and can be induced by many causes besides TA-GVHD, therefore the clinical diagnosis of the disease is difficult to make[15]. There is currently no effective treatment for this disease hence prevention is still the main measure to avoid TA-GVHD.

Table 3. Cytokine secretion of lymphocytes treated by MB+L/gamma-irradiation upon stimulation of PHA (pg/mL, n=7)

Cytokine	Untreated		γ-irradiation		MB+L	
	PHA-	PHA+	PHA-	PHA+	PHA-	PHA+
IFN-γ	10.154±17.749	6692.280±6816.743	16.560± 20.454	10165.254± 6820.270*	6.406±11.577	2539.429± 2499.843*
IL-1β	6.843±18.104	443.498±307.260	0.000±0.000	207.831± 181.996*	0.000±0.000	221.932± 244.532*
IL-4	0.719±1.336	10.528±24.022	0.979±1.669	30.742±36.668	11.708±28.381	12.588±20.897
IL-6	4.392±6.768	3913.118±3459.898	0.872±1.255	2061.429± 1377.848*	31.191±67.704	2163.261± 3962.139*
IL-8	1076.434±1412.202	22879.041±3937.404	404.674± 652.888	16068.186± 3637.098*	2125.808± 2534.489	15908.913± 4045.119*
IL-10	0.000±0.000	182.882±272.634	0.000±0.000	292.024± 303.664*	0.000±0.000	49.053± 115.049*
IL-12p70	1.501±3.971	2.388±4.099	4.399±9.783	3.104±5.305	0.684±1.703	0.069±0.184

*Levels of cytokines present in the supernatants of MB+L/γ-irradiation treated cells are significantly less than those present in the corresponding supernatants of untreated cells ($p<0.05$).

Table 4. Cytokine secretion of lymphocytes treated by MB+L/gamma-irradiation upon stimulation of anti-CD3+anti-CD28(pg/mL, n=6)

Cytokine	Untreated		γ-irradiation		MB+L	
	no stimulation	Anti-CD3+CD28	no stimulation	Anti-CD3+CD28	no stimulation	Anti-CD3+CD28
TNF-α	26.532±64.989	4299.938±2692.332	1.805±4.421	2385.508± 2883.461*	5.233± 12.819	1997.135± 3077.679*
IFN-γ	15.002±36.746	2002.782±725.260	1.445±3.540	1379.400± 1443.759*	0.480±1.067	745.948± 1019.539*
IL-1β	1.025±2.511	58.692±51.511	0.767±1.878	27.695±36.292	0.062±0.151	28.507± 37.466*
IL-4	0.033±0.082	17.597±23.691	0.085±0.208	54.902±51.327	0.043±0.106	11.260±13.215
IL-6	5.587±10.821	400.388±335.097	10.595± 20.634	152.832± 209.304	0.000±0.000	71.358± 143.744*
IL-8	893.162±1120.530	16403.267±13608.514	872.038± 1067.472	2376.616± 970.131*	891.118± 1016.303	2723.187± 561.085*
IL-10	2.600±5.686	482.952±344.522	4.620± 10.219	153.564± 206.730*	0.076±0.170	70.496± 154.424*
IL-12p70	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000

*Levels of cytokines present in the supernatants of MB+L/γ-irradiation treated cells are significantly less than those present in the corresponding supernatants of untreated cells ($p<0.05$).

Gamma-irradiation is widely recognized for TA-GVHD prevention by inhibition the proliferation of allogeneic

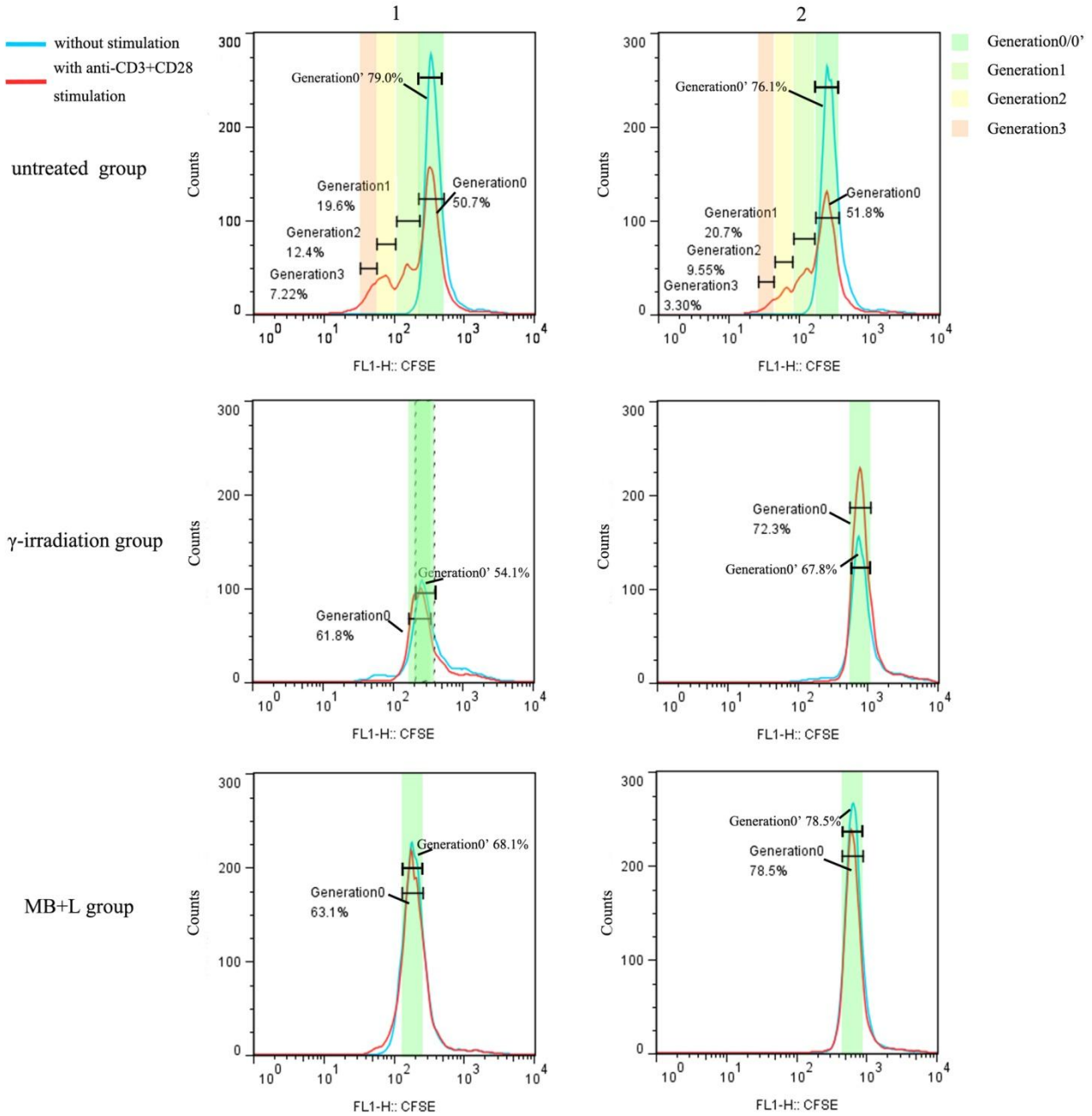

Fig. 2 CFSE-labeled lymphocytes of untreated, gamma-irradiated and MB+L treated groups were stimulated by anti-CD3+CD28 antibodies for 72h, and cell divisions were analyzed by flow cytometry. Blue lines represented generations without stimulation, while lymphocytes stimulated by anti-CD3+CD28 antibodies were shown with red lines. Each cell generation and its proportion of total CD3+ live cells were also shown in the figure.

lymphocytes. Due to limitations on equipment use, staffing and irradiation hazard, not all blood products should accept irradiation. Therefore the prevention of disease in clinical practice depends largely on patients' physician to determine. It was confirmed that TA-GVHD not only occurred in immunocompromised patients but also in HLA-mismatched immunocompetent patients, which make it more difficult for doctors to define[16]. So there is still risk of TA-GVHD beyond the cases demanding irradiated blood transfusion. In this situation, if each product was processed by pathogen inactivation technology, the probability of TA-GVHD occurrence can be minimized.

Currently the reported lymphocyte inactivation studies mainly adopted psoralen with ultraviolet light source of UVA or riboflavin with UVB/UVA. Studies have shown that these methods can cause damage to certain blood components including proteome changes and platelet lesions[17, 18]. Besides ultraviolet light itself may affect platelet function[19, 20].

Methylene blue is a phenothiazine dye, and its absorption

spectrum shows that MB has two absorption peaks in visible light, which are located at 620nm and 665nm, respectively (Fig. 1). Serving as a clinical plasma viral inactivation method, MB+L has been applied for years in China and several European countries. Its efficacy and safety have been confirmed. Upon visible light excitation, MB can cause damage to pathogen nucleic acids, thereby inhibiting replication of pathogens. According to this mechanism, in theory, this method is also applicable to the inactivation of lymphocytes so as to achieve the purpose of TA-GVHD prevention.

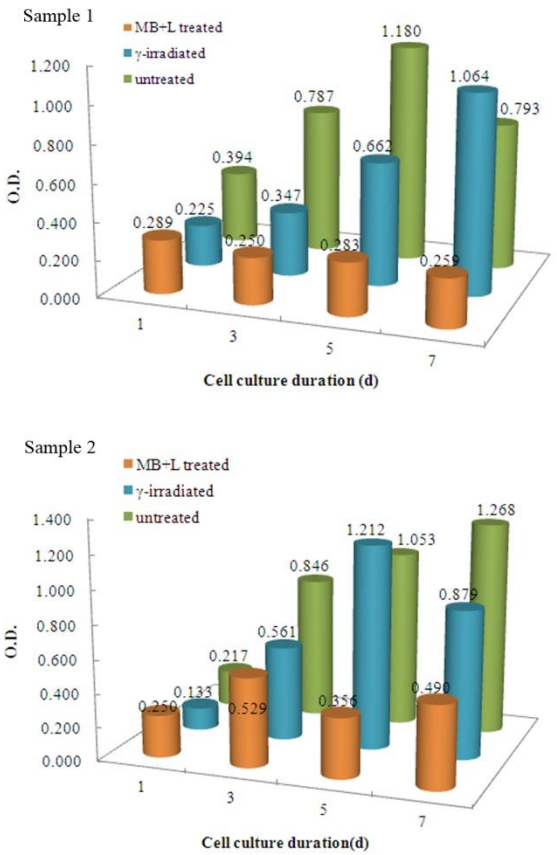

Fig 3. The figure showed that during 7 days of incubation with PHA, untreated and gamma-irradiated lymphocytes proliferated significantly, while MB+L treated cells showed barely any proliferation.

In this research, we studied the inactivation effects of MB+L on human peripheral lymphocytes. We used a final concentration of 3μmol/L MB for photochemical treatment, which is less than three percent of the clinical dose for curing nitrite poisoning (1-2mg /kg body weight). Besides we extend the exposure time of MB+L to 50min in order to enhance photochemical intensity. We also assess RBCs functions after MB+L treatment to evaluate MB+L effects on RBCs. The results (not shown) showed that in the condition we finally used to inactivate lymphocytes, there were no significant differences between untreated RBCs and PDT treated RBCs, except for K+ leakage. The reason for K+ leakage we suppose might be related to the inhibition of Na, K-ATPase activity which also happened in gamma-irradiation treated cells. The real cause is being studied through which we wish to find a way to protect the function of Na, K-ATPase.

Based on the pathogenesis of TA-GVHD, the ability of cell proliferation is essential for disease development. If lymphocytes were unable to proliferate, the attack on recipients would be greatly suppressed and limited. By BrdU DNA incorporation method, it showed that after PHA stimulation the proliferation ability of MB+L/ gamma-irradiation treated cells were significantly inhibited compared to untreated ones. Furthermore, the inhibition rate calculated according to the formula listed above indicated that MB+L treatment has better inhibitive effects on cell proliferation. Because of the high proliferation of untreated group upon anti-CD3+anti-CD28 stimulation, BrdU incorporation method was not suitable for proliferation detection in this case. Thus we used CFSE cell labelling test to measure cell divisions of lymphocytes. Results have shown that, unlike untreated groups with 3 successive generations, MB+L/ gamma-irradiation treated cells had no divisions indicating that cell proliferation ability was inhibited. Also we found that there may be a slight reduction in fluorescence intensity of gamma-irradiated cells which might be caused by influence on the intracellular esterase activity thereby blocking conversion of CFSE to fluorescence substances. The exact reason needs to be verified. Results also showed that MB+L treated lymphocytes presented barely any proliferation during prolonged incubation with stimuli indicating that MB+L treatment is irreversible for lymphocyte inactivation.

Studies have shown that, besides the direct organ damage mediated by T cell, the large number of inflammatory cytokines such as interleukin (IL), tumor necrosis factor (TNF) and interferon (IFN) are also involved in the occurrence and development of GVHD[21, 22]. These cytokines can further activate effector T cells to amplify leading to enlarge of the immune effects which exacerbate the pathological damage on recipients. We examined the levels of cytokines present in culture supernatants before and after PHA or anti-CD3+anti-CD28 stimulation. By statistical analysis, the suppressed secretion of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ from MB+L/gamma-irradiation groups showed that cytokine production of lymphocytes was inhibited by both of the treatments. Some cytokines like IFN-γ and IL-10 of MB+L group were even lower than those of gamma-irradiation group, which indicated MB+L treatment showed at least equivalent, sometimes maybe even higher abilities in TA-GVHD prevention than gamma-irradiation. Although none statistical differences of IL-4 and IL-12 were found between untreated group and MB+L/gamma-irradiation groups, since the low level present in supernatants, these two cytokines may make little impact on GVHD development if it was the case in vivo.

Following full assessment of RBCs functions, we tend to demonstrate the in vivo effects of MB+L for TA-GVHD prevention in mouse model to evaluate the efficiency and safety of MB+L for blood treatment.

Conclusions

The results of this study suggest that in addition to inactivating virus, MB+L treatment is able to functionally inactivate lymphocytes from proliferation and cytokine secretion. By comparison with gamma irradiation method, the effect of MB+L on lymphocyte was proved to be valid. According to this, we

propose that MB+L treatment may provide an alternative to gamma irradiation for preventing TA-GVHD in the future. To achieve this goal, we will further assess the impact of MB+L photochemical effects on red blood cells and platelets, and to provide in vivo data for evidence of effectiveness and safety.

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Notes

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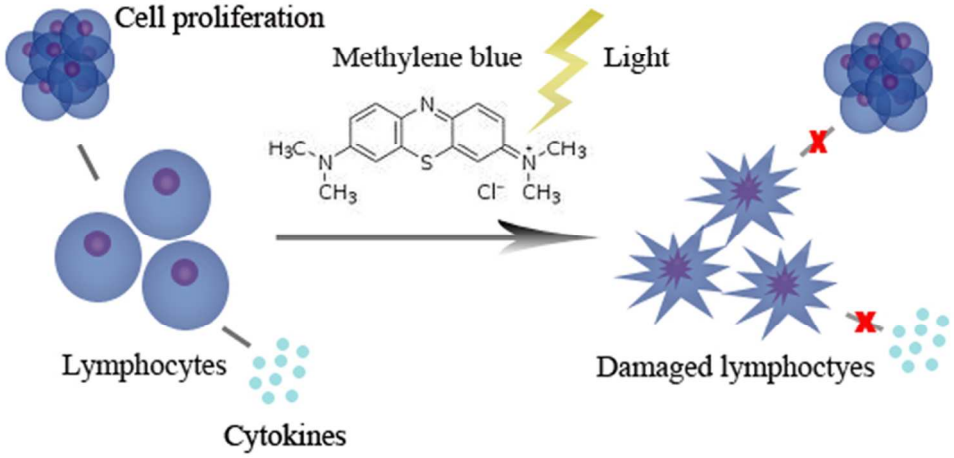

Upon visible light excitation, MB can cause damage to pathogen nucleic acids, thereby inhibiting lymphocyte proliferation and cytokine secretion.
70x38mm (300 x 300 DPI)