KN035 MLR Results

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Abstract

The mixed lymphocyte reaction (MLR) occurs between two allogeneic lymphocyte populations which, while being ex vivo cultivated, can mutually stimulate T cell proliferation primarily due to the difference in D and DP region in major histocompatibility antigens (HLA). The more difference between the two HLAs, the stronger response is observed.

A MLR system is construted for this project. First, DC cells are differentiated from PBMCs and then stimulated to mature. Such DC cells are used as Antigen Presenting Cells (APC). CD4+ T cells then separated from PBMCs of another individual are used as allogeneic T cell receptor. Under coculture, APC (DC) is able to present the surface major histocompatibility complex (MHC) or antigen-MHC composite to allogeneic T cell receoptor (CD4+ T cell), inducing CD4+ T cell activation. Activated T cells will secrete IFN-γ. PD1 expressed on the surface of T cells and PDL1 expressed on the surface of DC cells can constitute a signaling pathway which negatively regulates the activation of T cells and accordingly IFN-γ secretion. In this work, activating effect of KN035 on CD4+ T cells that were stimulated by allogeneic antigens in MLR was evaluated by measuring the IFN-γ expression level in the supernatant of the co-cultivated DC and CD4+ T cells with the presence of KN035.

In this test, 2.41H9OP(Durvalumab, AZ/Medimmune), an anti-PDL1 antibody from AZ/Medimmune, was used as a parallel control. The test results showed that the extent to which KN035 promoted IFN-γ-secreting ability of CD4+ T cells was in proportion to KN035 concentration. Under the same concentration, KN035 has showed a slightly better performance than 2.41H9OP in activating T cells.

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1. PURPOSE

The purpose is to test and compare the activating effects of KN035 and 2. 41H9OP on $CD4^+$ cells that are stimulated by allogeneic antigens in MLR.

2. PROCEDURE

2.1. Test sample

Sample name	Lot#	Manufacturer	Concentration
KN035	141230-15020601	Aphamab Co Ltd (Suzhou, China)	18.978mg/ml
2.14H9OP	TE20140915	Aphamab Co Ltd (Suzhou, China)	3.7mg/ml

2.2. Reagents and supplies

RPMI-1640(Gibco, 11875-135)

FBS(Gibco: 16000-044)

TNF-α (PrimeGene, Shanghai, 103-1)

GM-CSF (PrimeGene, Shanghai, 102-03)

IL-4 (PrimeGene, Shanghai,101-04)

CD4 T CellIsolation Kit (MiltenyiBiotec, 130-096-533)

Human IFN-γ ELISA Ready-SET-Go!® (eBioscience, 88-7316-88)

96-well round bottom cell culture plate (Costar，3799)

2.3. Experiment Procedure

2.3.1. DC cell preparation

1) PBMC 4# are thawed and resuspended in serum-free RPMI1640. After being cultivated for 1-2h in a 10cm cell culture dish, unadhered cells are removed, and the cells are cultured in RPMI1640 medium with the presence of 10% FBS. Fresh medium containing 50ng/mL GM-CSF and 25ng/mL IL-4 is fed every 2~3 days.
2) After 6 days, 50ng/mL TNF-αis used to mature the cells for 24h. DC cells are then collected and counted via centrifugation (cells remaining adherent to the wall after centrigugation are digested by trypsin).
3) Add 1×10^4cells /well into the U-shaped bottom of 96-well plate containing 50μL/well serum-free medium. Stand for 2-4h, allowing cells to expand and adhere to the wall.

2.3.2. CD4+ T cell preparation

1) Thaw PBMC 5#.
2) After centrifugation, remove the supernatant.
3) Resuspend the cells with 40ul MACS buffer.
4) Add 10μL/well CD4+ T cell biotinylated antibody and homogenize the mixture. The cells are inculated for 5min under 4°C.

5) Add 30μL/tube MACS buffer.

6) Add 20μL/tube CD4+ T cell MicroBead, and incubate the tube for 10min at 4°C.

7) Add 3mL MACS buffer to rinse the column.

8) Add the aforementioned cell suspension, and then add 3ml MACS buffer to wash and collect the CD4+ T cells.

9) Centrifuge cells, remove the supernatant and perform cell counting.

2.3.3. MLR test

1) Add 1×10^5/well $CD4^+$ T cells that were separated using MACS onto U-shaped bottom of 96-well microplate pre-filled with 50μL 20% FBS.

2) Add antibodies diluted using RPMI1640+10% FBS into each well. Seven concentraions of antibodies are obtained by performing 10-fold serial dilutions starting from 40μg/mL. Three control groups are set as well: medium only, DC only, CD4+ T only.

3) Cultivate the cells in the incubator for 5 days. The supernatant is collected and IFN-γ content is measured using Human IFN-γ ELISA Ready-SET-Go!®.

2.3.4. IFN-γ level test

1) Add 100μL/well capture antibody that was 1:250 diluted using 1× Coating Buffer, store the plate at 4°C overnight.

2) Wash the plate 5 times with 300μL/well wash buffer (1× PBS, 0.05% Tween20), pat dry the plate after the last wash.

3) Block the wells by adding 300μL 1× ELISA/ELISPOT Diluent that was 1:10 diluted using purified water. Incubate the plate at room temperature for 1h.

4) Wash the plate 5 times.

5) Dilute standard samples with 1× ELISA/ELISPOT Diluent, and add into each cell 100μL diluted standard samples. The highest concentration is 32ng/mL, and a total of 11 concentrations are obtained through 2-fold serial dilutions. 100μL 1× ELISA/ELISPOT Diluent is used as blank control. Incubate the plate at room temperature for 2h.

6) Wash the plate for 5 times.

7) Add 100μL detection into each well. Antibody that was 1:250 diluted with 1× ELISA/ELISPOT Diluent, incubate the plate at room temperature for 1h.

8) Wash the plate for 5 times.

9) Add into each well 100μL Avidin-HRP that was 1:250 diluted with 1× ELISA/ELISPOT Diluent, incubate the plate at room temperature for 30min;

10) Wash the plate for 7 times;

11) Add 100μL/well 1× TMB Solution; then the plate is incubated at room temperature for 7min;

12) Add 50μL/well Stop Solution, OD value was read at 405nm and 570nm.

2.4. Experiment Results

2.4.1. Data processing method

The final optical densities (OD) are obtained by subtracting OD at 570nm from OD at 450nm. Logarithm values of sample concentrations in the standard curve are fitted with measured OD using a Four Parameter Logistic Fit function in Softmax Pro. The as-obtained four-parameter equation is applied in calculating the concentrations of test samples. All the processes are completed by the software. The resulting data is plotted using GraphPad Prism6.

2.4.2. Test results

Table 1. Plate layout illustrating well assignment

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard curve(pg/ml)											
	32000	16000	8000	4000	2000	1000	500	250	125	62.5	31.25	0
B	2.41H9OP 20ug/ml				KN035 20ug/ml							
C	2.41H9OP 2ug/ml				KN035 2ug/ml							
D	2.41H9OP 0.2ug/ml				KN035 0.2ug/ml							
E	2.41H9OP 0.02ug/ml				KN035 0.02ug/ml				DC+CD4			
F	2.41H9OP 0.002ug/ml				KN035 0.002ug/ml				DC+CD4			
G	2.41H9OP 0.0002ug/ml				KN035 0.0002ug/ml				DC+CD4			
H	2.41H9OP 0.00002ug/ml				KN035 0.00002ug/ml							

Table 2. OD results ($OD=OD_{450}-OD_{570}$)

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.11	3.057	3.0583	3.01	2.596	1.676	0.902	0.4609	0.2073	0.1117	0.0538	0.0142
B	0.604	0.8707			1.457	0.8593						
C	1.2228	0.5678			0.72	1.2321						
D	1.4735	2.7547			1.473	1.3271						
E	0.7075	1.4943			1.956	2.6212			2.7597			
F	1.5199	1.6177			2.685	1.2676			0.5169			
G	2.6332	0.4856			0.899	2.4366			1.263			
H	2.857	1.4796			2.958	2.0271						

2.4.3. Results analysis

Table 3. IFN-γ expression in CD4+ T cells stimulated by KN035/2.41H9OP (average value)

	IFN-γ Expression(ng/ml)	
Concentration（μg/ml)	2.41H9OP	KN035
20	8.449	12.7135
2	10.0275	10.7945
0.2	17.646	7.5905
0.02	3.0575	8.4615
0.002	1.7245	3.1205
0.0002	2.566	2.2845
0	0.9995	0.9995

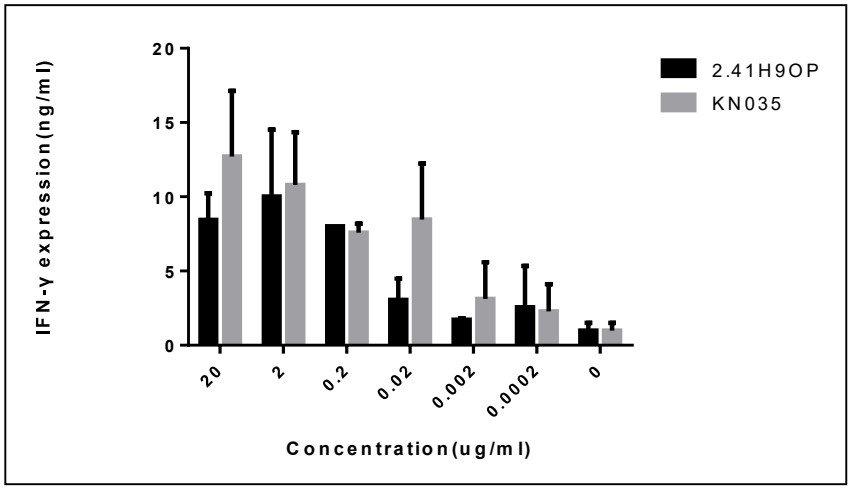

Figure 1. Histogram of IFN-γ expression in $CD4^+$ T cells stimulated by KN035 or 2.41H9OP

3. CONCLUSION

KN035 protein was able to promote IFN- γ -secreting ability of CD4+ T cells in proportion to KN035 concentration. Under the same concentration, KN035 showed a slightly better performance than 2.41H9OP in activating T cells.