CHAPTER V

RESULTS

5.1 Determination of sandwich ELISA optimal conditions used for the detection of antibodies in cell supernatants.

a. Block titration of anti human IgG+IgM (H+L) coated plate

Five concentrations of antihuman IgG and IgM were coated on an ELISA plate. Serial dilutions of standard IgM were added. The peroxidase conjugated anti human IgG and IgM at dilition 1: 5,000 and 1:10,000 were used. It was found that anti human IgM+IgG at a concentration of 0.2 μg/ml gave the best result and was selected for further experiments.

a.

Peroxidase conjugated Ab dilution 1:5,000 / Std. IgM (pg/ml)	OD mean ± SD		
	Anti human IgG+IgM (H+L) μg/ml		
	0.2	1	5
100	2.189 ± 0.096	2.765 ± 0.000	2.852 ± 0.000
10	1.562 ± 0.078	1.992 ± 0.017	2.082 ± 0.013
1	1.512 ± 0.077	1.952 ± 0.005	2.066 ± 0.136
0.1	0.627 ± 0.021	0.891 ± 0.022	1.520 ± 0.013
Neg. control	0.070 ± 0.004	0.074 ± 0.001	0.078 ± 0.001

b.

Peroxidase conjugated Ab dilution 1:10,000 / Std. IgM (pg/ml)	OD mean ± SD		
	Anti human IgG+IgM (H+L) μg/ml		
	0.2	1	5
100	0.909 ± 0.008	1.718 ± 0.088	1.695 ± 0.080
10	0.893 ± 0.008	1.673 ± 0.080	1.675 ± 0.080
1	0.861 ± 0.021	1.488 ± 0.089	1.529 ± 0.007
0.1	0.354 ± 0.003	0.626 ± 0.015	0.971 ± 0.006
Neg. control	0.070 ± 0.004	0.074 ± 0.001	0.078 ± 0.001

Table 1. Block titration of anti human IgG+IgM (H+L) coated plate. Anti human IgG+IgM (H+L) at concentrations of 0.2, 1, and 5 μg/ml were tested. The experiments were performed in triplicate. Data showed the results of using peroxidase conjugated antibody at dilution of a. 1:5000 and b. 1: 10,000. The negative control means no standard IgM added.

b. Titration of peroxidase conjugated anti human IgG+IgM (H+L)

To find the optimal condition of concentration of the peroxidase conjugated anti human IgG+IgM (peroxidase conjugated Ab), serial dilutions of this antibody were tested. The LP-1 plasma cell line secretes IgM antibody. The peroxidase conjugated Ab, at a dilution of 1:20,000, gave the highest O.D., when compared to 1:40,000 and 1:80,000. Despite 1:40,000 and 1:80,000 giving a good lower O.D background, the sensitivity was lower than the 1:20,000. When considering a low

concentration of antibody secretion in the supernatants, with low cell numbers in the experiments, the dilution of peroxidase conjugated Ab 1:20,000 was selected for further experiments, as it was more sensitive and gave a positive value. The experiments were repeated twice and gave a similar outcome.

a.

Peroxidase conjugated Ab dilution / Std IgM (pg/ml)	OD mean ± SD	
	1: 20,000	1: 40,000
100	2.119 ± 0.077	1.841 ± 0.131
50	1.508 ± 0.059	0.771 ± 0.020
25	0.679 ± 0.032	0.346 ± 0.006
12.5	0.396 ± 0.018	0.214 ± 0.006
Neg. control	0.106 ± 0.061	0.090 ± 0.013

b.

Peroxidase conjugated Ab dilution / Std IgM (pg/ml)	OD mean ± SD		
	1: 20,000	1: 40,000	1: 80,000
100	2.110 ± 0.080	1.465 ± 0.056	0.752 ± 0.013
50	1.626 ± 0.015	0.864 ± 0.005	0.448 ± 0.002
25	0.931 ± 0.046	0.462 ± 0.008	0.253 ± 0.004
12.5	0.505 ± 0.013	0.265 ± 0.003	0.161 ± 0.018
Neg. control	0.103 ± 0.020	0.072 ± 0.001	0.078 ± 0.011

Table 2. Titration of peroxidase conjugated anti human IgG+IgM. Three dilution of enzyme conjugated antibody, 1:20,000, 1:40,000 and 1:80,000 were tested. The experiment were performed in triplicate and repeated twice a. and b.

c. Sensitivity of ELISA test for the detection of antibody.

Standard IgM concentrations between 50-3.25 pg/ml were used to determine the sensitivity test. LP-1 culture supernatant containing IgM antibody was used as a positive control in order to find any background from a cell culture medium. It was found that the ELISA condition could detect IgM as low as 6.25 pg/ml

Std IgM (pg/ml)	OD Mean ± SD
50	0.816 ± 0.014
25	0.402 ± 0.017
12.5	0.227 ± 0.002
6.25	0.151 ± 0.001
3.12	0.118 ± 0.005
Neg. control (PBS)	0.115 ± 0.028

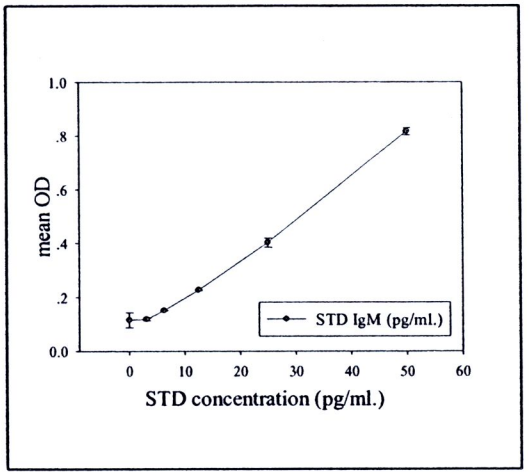


Table 3. Antibody standard in the concentration of 50, 25, 12.5, 6.25 and 3.12 were determined by using ELISA. The amount of antibody was presented in an O.D mean of triplicate. The O.D of the negative control, without supernatant, was 0.115+0.028. Data also showed in standard curve.

5.2 Detection of antibody production in cell culture supernatants by ELISA

To verify that the Raji cells were activated and differentiated into antibody producing plasma cells, supernatants harvested from PWM+IL-2 stimulated Raji B cells were tested for antibody secretion. Despite low amounts, antibodies gradually increased in supernatants of PWM stimulated Raji B cells starting from day 3 to day 9. In unstimulated B cell control, a low amount of antibody could be detected, but not

increase in the time of stimulation. This might be due to some substances in culture medium that can trigger Raji B cells, but might not be able to drive the full differentiation. Supernatant dilutions of 1:2 and 1:4 show similar results, but the dilution of 1:2 gave a clearer result. In conclusion, the stimulation was successful and the cells differentiated into plasma cells, as shown by secreting antibodies.

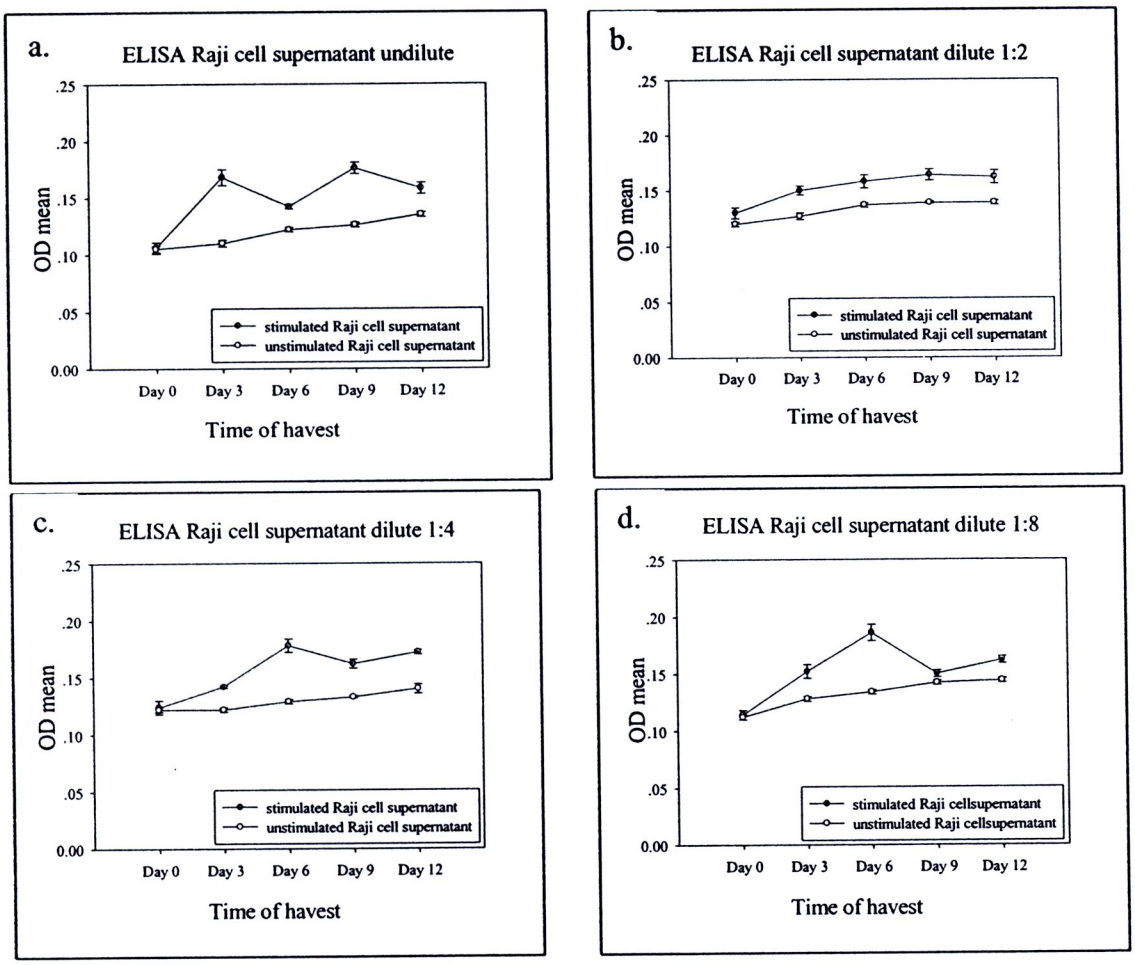


Figure 5. Antibody secretion from PWM+IL-2 stimulated Raji B cells at different time points. Cell culture supernatants were harvested on day 0, 3, 6 and 9 and 12 antibody secretion was determined by using ELISA. The amount of antibody was presented in an O.D mean of triplicate. Supernatant dilution used a. undiluted b. 1:2 c. 1:4 d. 1:8. Standard IgM 3 concentrations and LP-1 supernatants at dilution of 1:2 and 1:4 were performed in parallel as internal controls. The O.Ds of LP-1 cell supernatants were 1.031±0.031 and 0.984±0.052 respectively and Standard IgM at 25, 12.5 and 6.25 pg/ml were 0.713±0.017, 0.364±0.036, and 0.211±0.047 respectively. The O.D of negative control, without supernatant, was 0.115±0.028.

Table 4. Antibody secretion from PWM stimulated Raji B cells at different time points. Cell culture supernatants were harvested on day 0, 3, 6 and 9 and 12 and antibody secretion was determined by using ELISA. The amount of antibody was presented in an O.D mean and S.D of triplicate. Supernatants were undiluted, 1:2, 1:4 and 1:8.

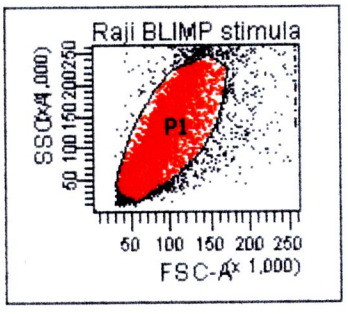
Day of harvest	mean OD ± SD							
	undiluted supernatant		dilution 1:2		dilution 1:4		dilution 1:8	
	stimulated	unstimulated	stimulated	unstimulated	stimulated	unstimulated	stimulated	unstimulated
Day 0	0.106 ± 0.005	0.105 ± 0.003	0.130 ± 0.005	0.120 ± 0.002	0.124 ± 0.006	0.122 ± 0.002	0.114 ± 0.004	0.112 ± 0.002
Day 3	0.168 ± 0.007	0.110 ± 0.003	0.150 ± 0.004	0.127 ± 0.003	0.142 ± 0.001	0.122 ± 0.002	0.152 ± 0.006	0.128 ± 0.002
Day 6	0.142 ± 0.002	0.122 ± 0.002	0.158 ± 0.006	0.137 ± 0.002	0.178 ± 0.006	0.129 ± 0.002	0.186 ± 0.007	0.134 ± 0.002
Day 9	0.176 ± 0.005	0.126 ± 0.002	0.164 ± 0.005	0.139 ± 0.001	0.162 ± 0.004	0.133 ± 0.001	0.150 ± 0.003	0.142 ± 0.002
Day 12	0.158 ± 0.005	0.135 ± 0.002	0.162 ± 0.006	0.139 ± 0.002	0.172 ± 0.002	0.140 ± 0.004	0.162 ± 0.003	0.144 ± 0.002

5.3 Staining of CD138 in PWM+IL-2 stimulated Raji cells

Raji cells were stimulated with PMW + IL-2 and the cells were harvested at different time points for staining of CD138, a plasma cell marker. The data were presented in the mean, median fluorescence intensity and change in percentage of cells that increase fluorescence intensity. Both unstimulated and stimulated cells showed the CD138 expression on day 3 and slightly increased on day 9, but stimulated cells were a little higher than the unstimulated cells. Expression of CD138 in Raji B cells might be due to some substances in culture medium that can trigger Raji B cells but these substances might not be able to drive the full differentiation. LP1 plasma cell line staining, a positive control, and the unstained and conjugated staining of Raji cells, which are negative controls, are shown in appendix.

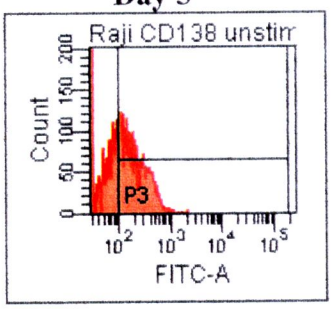
Dot plot of Raji cells

a.

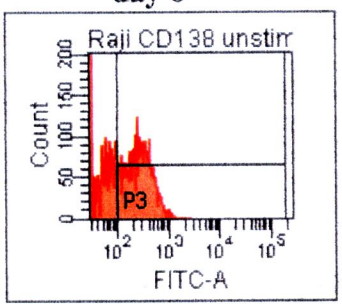


Unstimulated Raji cells

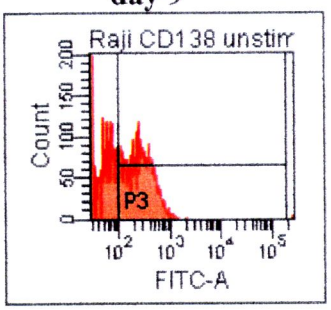
Day 3



day 6



day 9

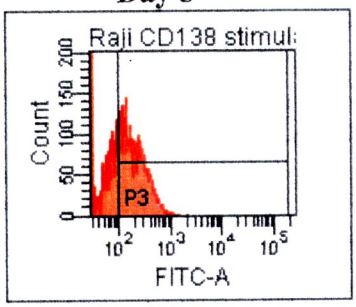


Fluorescent intensity

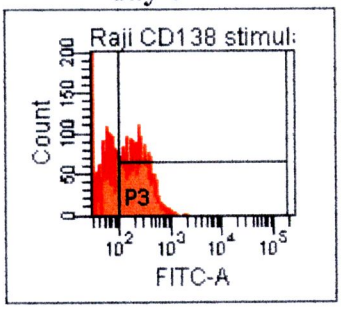
% Cell	57.6	56.4	52.5
Mean	245	289	244
Median	188	255	251

Stimulated Raji cells

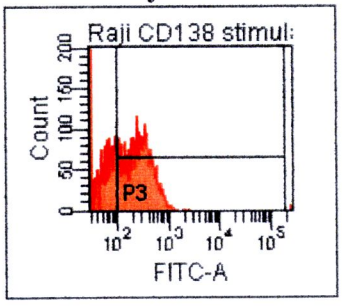
Day 3



day 6

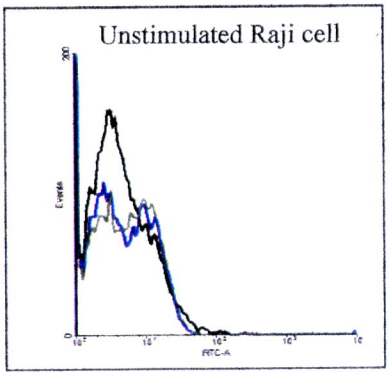


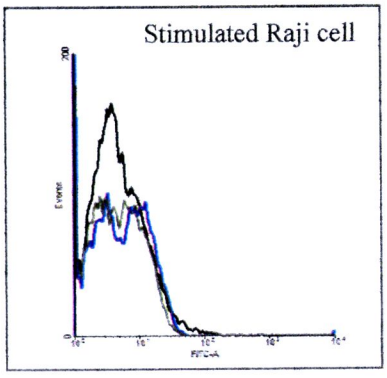
day 9



Fluorescent intensity

% Cell	52.4	54.7	60.7
Mean	132	263	305
Median	190	219	260





b.

Black: Raji cell day 3
Green: Raji cell day 6
Blue: Raji cell day 9

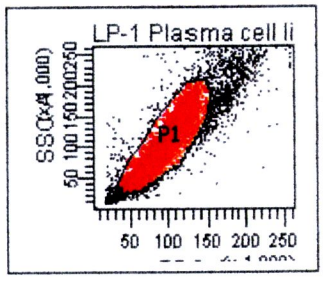
Figure 6. Expression of CD138 on Raji B cells by flow cytometry. Unstimulated and PWM+IL-2 stimulated Raji B cells were harvested and stained for a plasma cell maker on day 0, 3, 6 and 7 . a. individual histogram, b. over layer histogram.

5.4 Determination the optimal conditions used to stain Blimp-1 protein

5.4.1. Determination of permeabilized reagents used for protein Blimp-1 staining

To determine permeabilized reagents used for intracellular protein Blimp-1 staining, cold methanol and 0.1% Triton X-100 were compared for their capability of permiabilizing cells. LP-1, a plasma cell line, was stained with anti Blimp-1 antibody. Data was presented as a percentage of Blimp-1 positive cell change, and mean and median fluorescence intensity. No great difference between the two reagents was found, but cold methanol gave a slightly better result, as shown in Figure 7. Methanol was therefore used in further experiments.

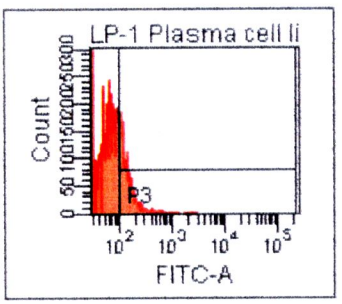
a.

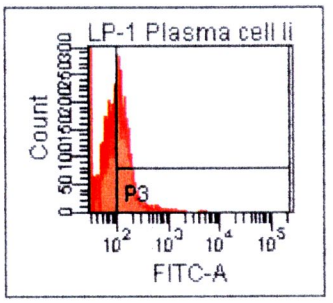


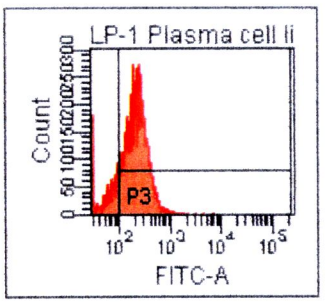
0.1 % Triton X-100

b.

Unstained | Conjugated control | Blimp-1







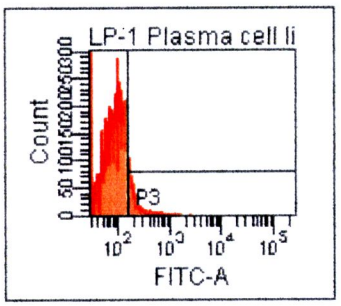
Fluorescent intensity

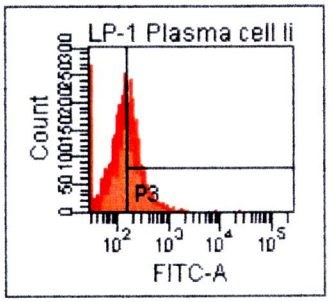
% Cell	18.9	39.0	80.8
Mean	178	188	246
Median	131	140	215

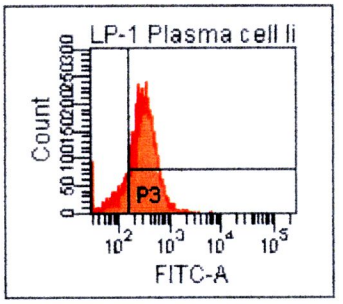
Cold methanol

c.

Unstained | Conjugated control | Blimp-1







Fluorescent intensity

% Cell	9.2	41.1	82.4
Mean	251	301	376
Median	131	229	322

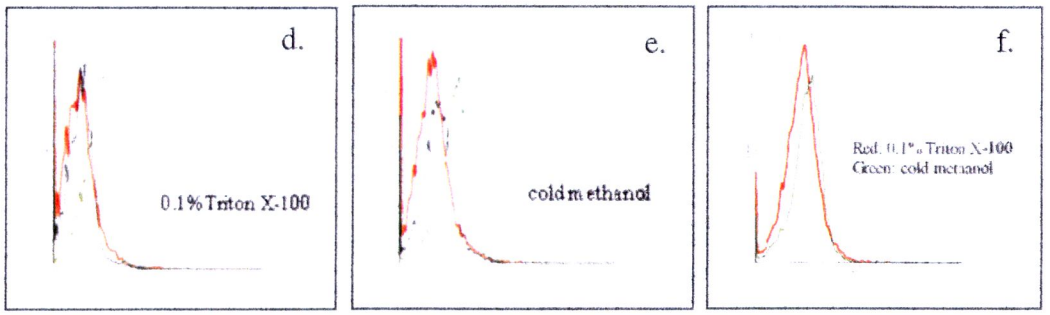
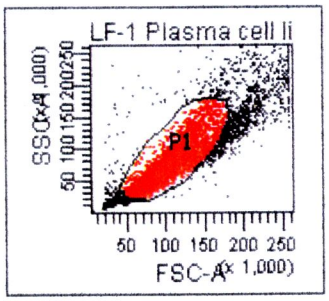


Figure7. Comparison of permeabilization reagents used for intracellular protein staining. Methanol and 0.1% Triton X-100 were used for permeabilization.

a. individual histogram, b. 0.1%Triton x-100, c. methanol d,e and f. overlayer of methanol and Triton x-100.

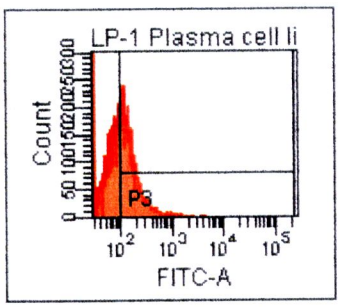
5.4.2. Titration of Blimp-1 antibody for staining

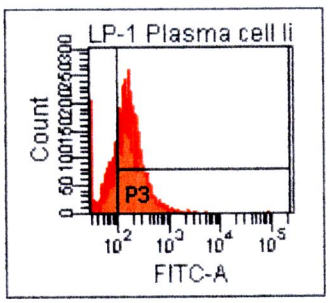
The LP-1 is positive for Blimp-1 and the Raji cell line, but a negative cell control for Blimp-1 were stained in parallel (see appendix). The staining procedure was performed in the same way as described above, but with cold methanol used as the permeability reagent. Cells were analyzed by a flow cytometer. There was no different in fluorescence intensity or change in percentage of Blimp-1 positive cells using either 1: 100 or 1:200 FITC conjugated anti goat IgG dilution. The conjugated control had the same background. Therefore, the dilution of 1:200 was used for further experiments. It was noticeable that the expression of Blimp-1 was not high even in a plasma cell line. It was considered that the change in Blimp-1 expression in Raji cells after stimulation might be difficult to detect. Staining anti Blimp-1 with a higher concentration (dilution 1:10) did not improve the intensity (data not shown).

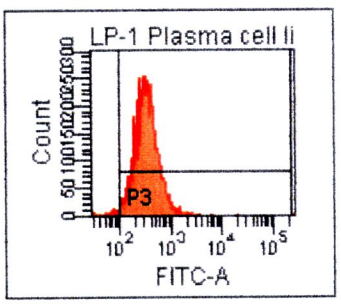


FITC conjugated anti goat IgG 1:100

a. Unstained Conjugated control Blimp-1





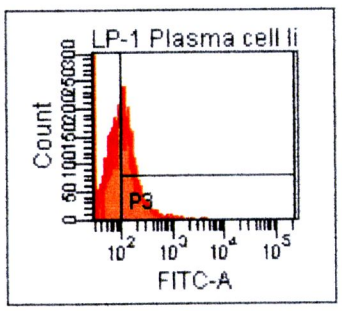


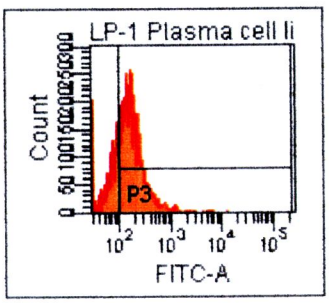
Fluorescent intensity

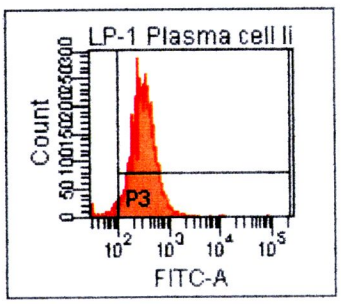
% Cell	21.5	50.1	92.7
Mean	251	301	376
Median	131	229	322

FITC conjugated anti goat IgG 1:200

b. Unstained Conjugated control Blimp-1



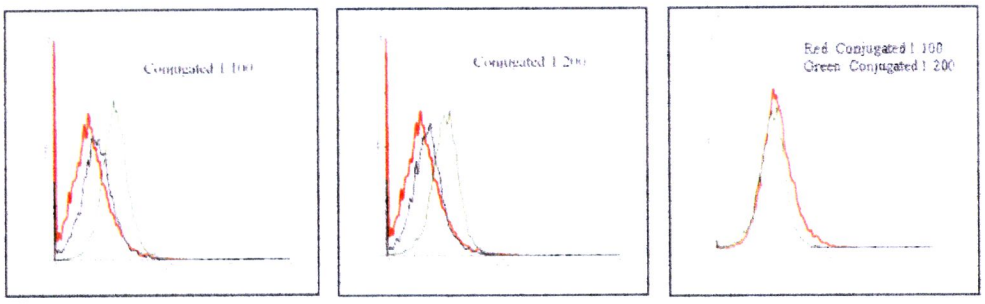




Fluorescent intensity

% Cell	21.5	47.5	90.1
Mean	251	301	376
Median	131	229	322

c.



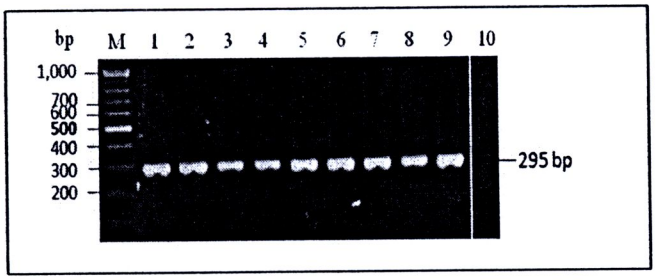
Red: unstain, Black: Conjugated control, Green: Blimp-1

Figure 8 Comparison of FITC conjugated anti goat IgG used for Blimp-1 staining. LP-1 cells were stained with goat anti blimp-1 dilution at 1:50 and stained again with FITC conjugated anti goat IgG at a dilution of a.1:100 and b.1:200 c. overlayer histogram.

5.5 Quality control in determination of Blimp-1 expression by using GAPDH gene control

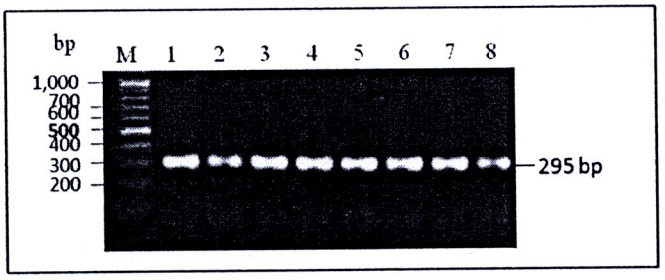
As the RNA isolation from samples wsas mall and unmeasurable, especially in later days when many of the cells died,the quality and amount of templates were determined by using GAPDH, a housekeeping gene, as a control. The experiments were repeated twice and gave the same results. All samples harvested at different time points showed a comparable amount of GAPDH gene, as shown in Figure 10. The amount of PCR products was presented in band intensities using program GeneTools from SynGene. Although there were some samples that had slightly different intensity bands, they had no consequence on the result of Blimp-1 isoform ratios because the Blimp-1 full length and Δ7 isoform ratio were compared in the same reaction and not among the cells harvested at different times.

a



Lane	1	2	3	4	5	6	7	8
Band density	1.24	1.20	0.97	1.00	1.21	1.26	1.19	1.06

b



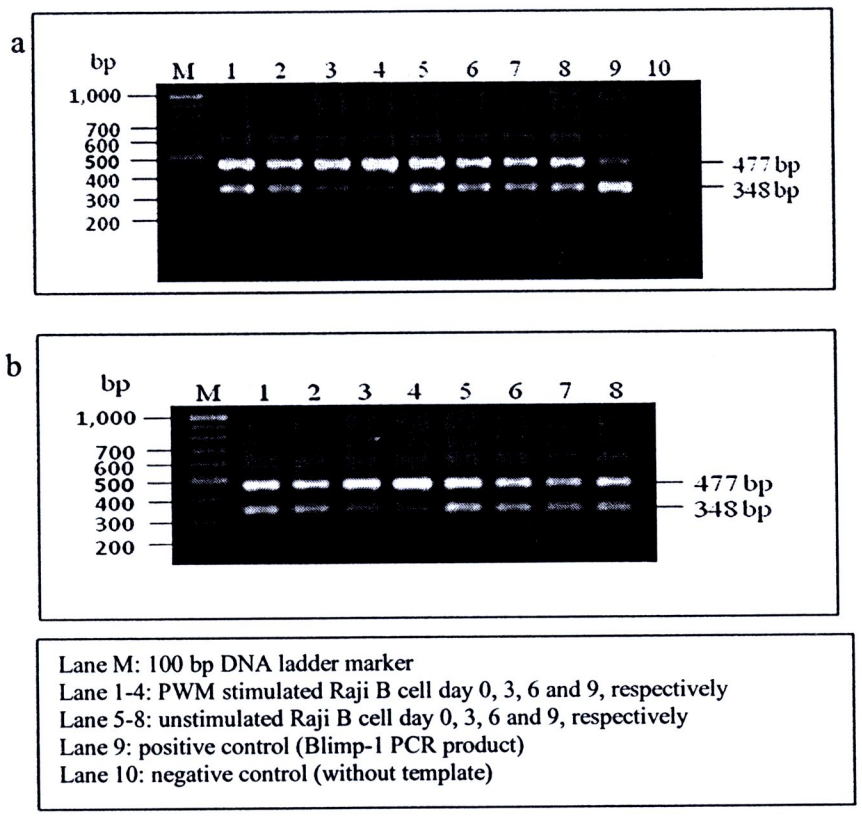
Lane	1	2	3	4	5	6	7	8
Band density	1.27	1.03	1.29	1.29	1.28	1.27	1.25	1.04

Lane M: 100 bp DNA ladder marker
Lane 2-4: PWM stimulated Raji B cells day 0, 3, 6 and 9, respectively
Lane 5-8: unstimulated Raji B cells day 0, 3, 6 and 9, respectively
Lane 9: positive control (GAPDH PCR product of 295 bp)
Lane 10: negative control (without template)

Figure 9. Determination of the GAPDH gene for the quality control of samples. Cells harvested at different time intervals were determined for the GAPDH gene by using RT-PCR. The PCR products were run on 2.5% gels and stained with ethidium bromide. They were compared and presented in the band intensity. The experiment was repeated twice a. and b.

5.6 Determination of Blimp-1 mRNA isoform expressions in PWM+IL-2 stimulated Raji B cells

The kinetic expression of Blimp-1 mRNA isoforms in PWM stimulated in Raji B cells was determined on day 0, 3, 6, 9 and 12 by using RT-PCR. The amount of PCR products were presented as band intensities. The intensity of bands between the full length and Δ 7 Blimp-1 isoforms was compared and presented in an intensity ratio During the first days of stimulation, the band intensity ratio of the full length and Δ7 Blimp-1 isoforms were 1.73 and they increased gradually to 15.0 on day 9. In unstimulated B cell control, the ratio was 1.56 on day 0 and increased slightly on day 3 and remained constant on day 6 and 9. In the steady state, the full length isoform expression was about 1.5-2 times greater than the Δ7 Blimp-1 isoform. Upon the differentiation to antibody secreting plasma cells, the full length Blimp-1 isoform was increasing to 14-15 times more than the basal level. The experiment was repeated twice and gave a similar result. (Table 9 and 10).



Lane M: 100 bp DNA ladder marker
Lane 1-4: PWM stimulated Raji B cell day 0, 3, 6 and 9, respectively
Lane 5-8: unstimulated Raji B cell day 0, 3, 6 and 9, respectively
Lane 9: positive control (Blimp-1 PCR product)
Lane 10: negative control (without template)

Figure 10. Kinetic expression of the full length and Δ7 Blimp-1 mRNA isoforms in PWM stimulated Raji B cells. The PCR products of full length and Δ 7 Blimp-1 isoforms were presented in band intensity ratios. The experiment was performed twice as shown in a. and b.

Day	Stimulated Raji cells				Unstimulated Raji cells			
	0	3	6	9	0	3	6	9
Full length isoform	1.14	0.93	1.13	1.35	1.14	0.88	0.71	0.84
Δ 7 isoform	0.66	0.43	0.15	0.09	0.73	0.51	0.41	0.50
Ratio	1.73	2.16	7.53	15.0	1.56	1.73	1.73	1.68

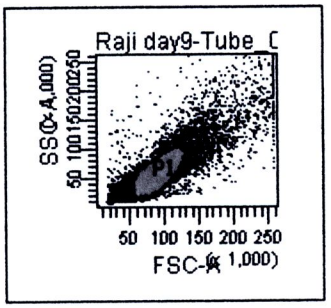
Day	Stimulated Raji cells				Unstimulated Raji cells			
	0	3	6	9	0	3	6	9
Full length isoform	1.20	0.90	1.24	1.41	1.31	0.87	0.52	0.74
Δ7 isoform	0.61	0.42	0.19	0.10	0.73	0.39	0.28	0.38
Ratio	1.98	2.16	6.57	14.13	1.80	2.25	1.87	1.95

Table 5. Kinetic expression of the full length and Δ7 Blimp-1 mRNA isoforms in PWM stimulated Raji B cells. The PCR products were run on 2.5% gels and stained with ethidium bromide. The PCR products of Blimp-1 isoforms were compared and presented in the band intensity ratios. The experiment was performed twice and presented in a. and b.

5.7. Determination of Blimp-1 protein expression in PWM+IL-2 stimulated Raji cells

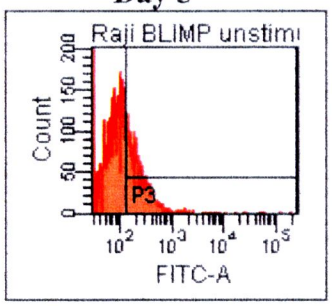
Raji cells, stimulated at different time interval; day 0, 3, 6, 9 were stained for Blimp-1 expression. The optimal condition and dilution of antibody were selected from the titration above. The staining process was performed in the same way as above. An LP-1 plasma cell line was stained in parallel as a control for Blimp-1 expression. The cells were then analysed by a flow cytometer. Data was presented as a percentage of Blimp-1 positive cell change and mean and median fluorescence intensity. Both unstimulated and stimulated Raji B cells expressed intracellular Blimp-1 at the basal level and slightly higher expression from 37.8 % to 54.3 % or 245 to 226 mean intensities after stimulation. When compared to unstimulated cells, a little more change of Blimp-1 expression in stimulated Raji cells upon the time was noticed. The result correlated with the expression of Blimp-1 at the mRNA level, which indicated how the 2 isoforms related to the function It should be noticed that this Blimp-1 antibody could not distinguish between the full length and Δ7 isoform proteins as in mRNA.

Blimp-1 detection in Raji cells harvested at different times

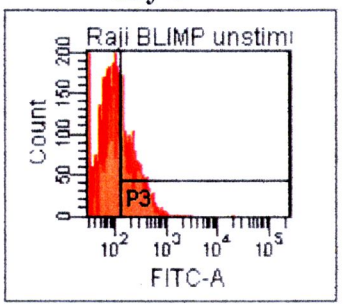


Unstimulated Raji cells

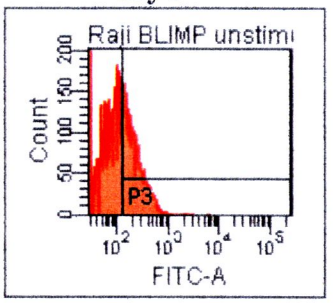
Day 3



day 6



day 9

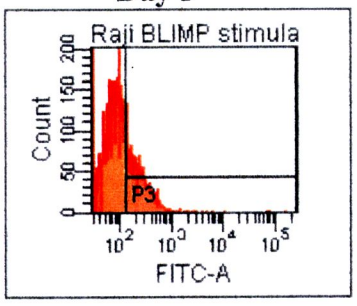


Fluorescent intensity

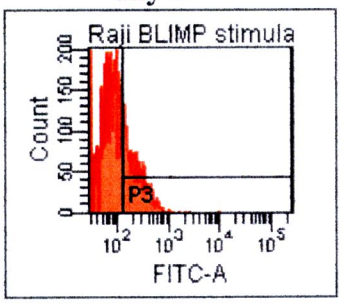
% Cell	32.2	32.7	37.8
Mean	631	271	245
Median	198	213	197

Stimulated Raji cells

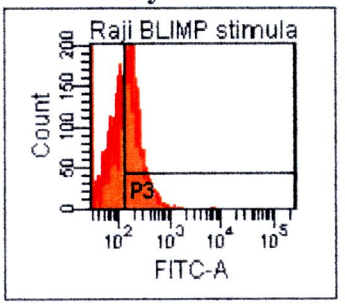
Day 3



day 6



day 9



Fluorescent intensity

% Cell	28.5	27.8	54.3
Mean	522	256	226
Median	209	206	189

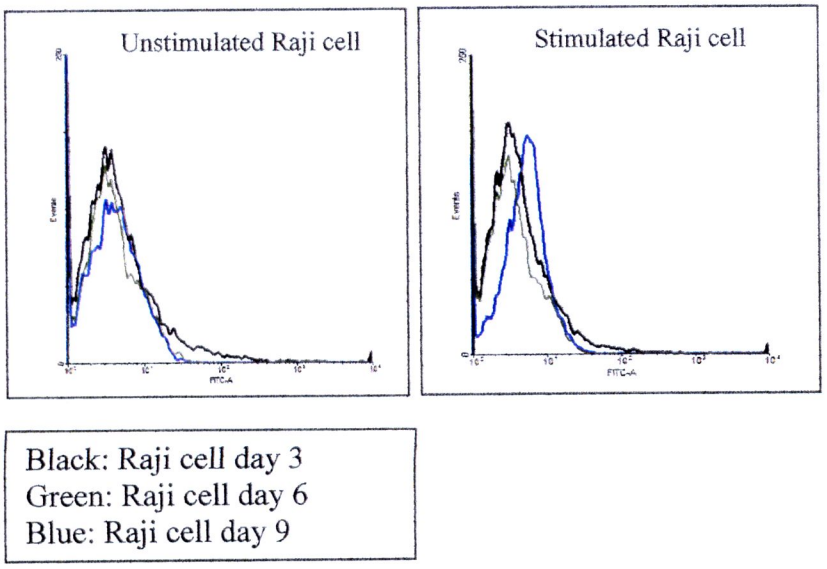


Figure 11. Blimp-1 protein expression in PWM stimulated Raji cells. Cells were fixed with 0.5% formaldehyde and then permabilized with cold methanol. They were stained firstly with goat anti Blimp-1 antibody at a dilution of 1:50 and secondly with rabbit anti goat IgG conjugated FITC dilution of 1:200 a. unstained, b. conjugated control, c. Blimp-1.