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A Comparison on Viability between CD4+ T cells and CD4+CD25+CD127- regulatory T cells by Excimer laser in the Peripheral Blood in vitro

조선대학교 대학원

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Excimer laser 조사 후 말초혈액 내 CD4+ T 세포와
CD4+CD25+CD127- 조절 T세포의 생존율 비교 연구

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목 차

표 목 차

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ABSTRACT

A comparison on viability between CD4+ T cells and CD4+CD25+CD127- regulatory T cells by excimer laser in the peripheral blood in vitro

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배경

많은 자가 면역 피부질환은 조절 T 세포의 역할이 중요하나 질환에 따른 역할이나 수의 차이가 많으며 역할도 다양하다. 최근에 조절 T 세포를 조절함에 있어 narrowband ultraviolt B(NBUVB) 광선치료가 아주 효과적인 것으로 알려져 있으나 정확한 기전에 대한 연구는 부족한 현실이다.

본 연구는 최신 NBUVB의 하나인 eximer laser로 T 세포를 조사한 후 직접적인 영향력을 확인하고자 한다.

방법

건강한 사람의 말초혈관에서 혈액을 채취 후 CD4+ T cell을 분리한 후 CD4+CD25-T 세포를 CD4+ T 세포로 CD4+CD25+CD127- T 세포를 조절 T 세포로 정의한 후 flow cytometry를 통해 분리한 후 24시간 동안 배양 후 excimer laser와 위장도구에 각각 조사한 후 T 세포간의 생존률을 확인하였고 나이와 성별, 등에 따른 결과의 연관성을 확인하였다.

결과

남자에서 CD4+ T 세포와 조절 T 세포의 생존률의 각각 64±4%, 77±3% 이었으며, 여자의 CD4+ T 세포와 조절 T 세포의 생존률의 각각 : 53±4%, 65±4%으로 남자에서 생존률이 높았으며 통계학적으로 유의하였다. 전반적으로 조절 T 세포가 CD4+ T 세포에 비해 엑시머 레이저 조사 후 생존률이 높았으며 이는 통계적으로 유의하였다. 상관변수분석을 통해 남성에서 CD8+ T 세포의 생존률이 높을수록 조절 T 세포의 생존률이 상승하는 결과를 보였다.

결론

우리의 연구결과는 조절 T세포가 CD4+ T 세포보다 직접적인 자외선 조사 후 발생되는 세포고사에 저항성을 나타내며 이 결과를 토대로 nbuvb 치료 후 조절 T 세포가 증가하는 이유를 설명할 수 있을 것이다. 본 연구의 제한점으로는 엑시머 레이저로 인한 세포고사의 다양한 기전들에 대한 연구가 결핍되어 있다는 것이다.

Key words : CD4+ T 세포, 조절 T 세포, Excimer laser

Ⅰ. Introduction

Regulatory T (Treg) cells are a subset of CD4+ T cells whose function is to suppress immune responses and maintain self-tolerance, therefore they have a central role in protecting an individual from autoimmunity.[1 Ultraviolet B (UVB) irradiation is a modality widely used for the treatment of different skin diseases such as psoriasis, vitiligo and other inflammatory dermatosis. It suppresses the immune system in several ways. UVB inhibits antigen presentation, induces the release of immunosuppressive cytokines, causes apoptosis of leukocytes, and induces Treg cells.[2,3]

It has been suggested that UVB irradiation of both active and resting peripheral lymphocytes in vitro induces programmed cell death (apoptosis) but not anergy of T cells.[4] Also, It has been reported that narrowband UVB (NBUVB) induces apoptosis of T cells within psoriatic lesions to resolve the lesions. It has already been reported that extracorporeal photopheresis and broadband UVB resulted in the expansion of Treg cells. Iyama et al. demonstrated by flow cytometry that NBUVB irradiation induces the increment of proportion of Treg cells.[6] Also Yamazaki et al. showed that exposure to UVB induced expansion of Treg cells up to 50-60% of the CD4+ T cells in the irradiated skin.[7] But, How Treg cells in the skin are expanded by UVB is poorly understood.

We hypothesized that there might be the difference of susceptibility to ultraviolet-induced apoptosis between Treg cells and conventional CD4+ T cells and the cumulative effect of high survival rate of Treg cells may contribute to the expansion of Treg cells in the UVB-irradiated skin. So, we investigated the viability of circulating CD4+CD25+CD127- Treg cells compared with conventional CD4+ T cells (except for Treg cells) after excimer laser irradiation in peripheral blood of normal volunteers in vitro.

Conventional CD4+ T cells in our study is defined as CD4+ T cells after deduction of Treg cells. We used excimer laser as the UV-emitting light sources in the induction of T cell apoptosis, because excimer laser is more effective in inducing T cell apoptosis in vitro than NBUVB irradiation and more convenient for use.[8,9]

II. Materials and Methods

Study group

The study was approved by the Ethics Committee of Chosun University Hospital, Gwangju, Korea. After obtaining written informed consent, 23 volunteers with no history of autoimmune diseases, chronic diseases, and systemic inflammatory diseases at present were enrolled. Samples of heparinized venous blood were obtained by venipuncture of the cubital vein from 23 healthy volunteers.

Cell count

Peripheral bloods (60ml) were prepared form 23 healthy volunteers. Flow cytometry was used for determination of Treg cells count, CD4+/CD8+ T cells ratio and CD8+ T cells/Treg cells. For Treg cells count defined as CD4+CD25+FoxP3/CD4+ T cells, peripheral blood mononuclear cells (PBMCs) were stained with antibodies against CD4, CD25, and FoxP3 in the Treg Detection Kit (Miltenyi Biotec, Auburn, CA, USA), and then subjected to flow cytometry on a FACScan flow cytometer (Beckman Coulter, Hialeah, FL, USA). Data were analyzed using ELITE Software (Coulter Corporation, Miami, FL, USA). Treg counts are expressed as percentage of CD4+T lymphocytes subpopulation in peripheral blood.

Cell separation

In Treg cells separation, PBMCs were separated as CD4+CD25+CD127- T cells with CD4+CD25+CD127- regulatory T cell Isolation Kit. And for conventional CD4+ T cell separation without Treg cells, some PBMCs were separated as CD4+CD25- T cells through negative selection with CD4+CD25+ regulatory T

cell Isolation Kit using AutoMACS separator (Miltenyi Biotec, Auburn, CA, USA).

UVB irradiation in vitro

308-nm xenon chloride excimer laser (XTRAC, PhotoMedex, Horsham, PA) was used as a light source. The same numbers (1 X 10^5 cells) of Treg, CD4+ T and CD8+ cells in 1ml of PBS in 12 well plates were prepared. Each same numbers of cells also were prepared for sham irradiation. After irradiation at a dose of 100mJ/cm^2 with excimer laser in vitro, each cells were incubated in at 37℃, 5% CO2 incubator for 24 hours in serum-free X-VIV015 medium (Lonza, Basel, Switzerland). For cell survival rate using flow cytometry, incubated cells were stained with annexin V and PI. We determined the survival rate (%) of cells as ratio of viable cells of excimer laser irradiation and sham irradiation.

Statistical analysis

Statistical analysis was conducted using SPSS18.0 for Windows (IBM corp,USA) and MedCalc 19.0.5. Data are presented as means ± s.e. Independent T-test were performed to compare the gender levels in age, FoxP3%, CD4 survival, Treg survival, CD4/Treg%, CD8 survival and CD4/CD8%. Multiple Paired T-test analysis was performed to compare the means in CD4 survival, Treg survival, CD8 survival. Correlation between Tregs variables were tested using the Spearman's rank correlation rho (ρ). And Multiple regression (stepwise selection) was performed to determine the effect of CD4 survival, CD8 survival and sex on Treg survival,

All tests were two-sided, and p values of less than 0.05 were considered significant, respectively.

III. Results

23 healthy volunteers (11 males and 12 females, with a mean age (34.57±1.68) were involved in this study. Flow cytometry was used for determination of FoxP3%, CD4+/Treg ratio and CD4+/CD8+ ratio. The frequency of Treg cells, calculated as the percentage of FoxP3+ cells was 3.51±0.31. The overall survival rate of Treg cells (71±3%) to excimr laser irradiation was higher than CD4+ cells (58±3%) and CD8+ cells (64±3%) (Table 1). Multiple Paired t-test was performed for mean comparisons between Treg survival, CD4 survival, and CD8 survival. Bonferroni adjustment was applied to obtain multiple p-values. That is, the significance level was 0.0167. All of the results were statistically significant (Table 2). Treg survival was the highest, followed by CD8 survival and CD4 survival (Figure 1).

The mean survival rate of males (Treg cell: 77±3, CD4+ cell: 64±4%, CD8+ cell: 68±4%) was high compared to females (Treg cell: 65±4%, CD4+ cell: 53±4%, CD8+ cell: 61±5%). We did the independent 2-sample parametric t-test for the survival rate difference of T cell subsets according to sex. The results of the Treg survival rate test by sex showed that the t statistic value was -2.335 and the p-value was less than the significance level 0.05 (5%) ($p = 0.030$). Treg survival rate was 77% for males and 65% for females. That is, Treg survival rate was higher in males than females (Table 3).

Correlation analysis was performed to find out whether there was any correlation between two variables. As a result, Spearman's rank correlation coefficient and significance probability are shown in the above table. There was a positive correlation between CD4+T cell and Treg cell survival rate ($\rho = 0.728$, $p < 0.01$) and between CD4+ and CD8+ T cell survival rate ($\rho =$

0.877, p <0.01) and a negative correlation between CD4+ T cell survival rate and CD4+/Treg (P = -0.567, p = 0.005, p <0.05). There was a positive correlation between CD8+ T cell and Treg cells survival rate (ρ = 0.760, p <0.01) and no correlation between CD8+ T cell survival rate and CD4 / Treg (ρ = -0.357, p = 0.097> 0.05) (Table 3, Figure 2). These results mean that the survival rate of T cell subsets might be closely connected with each other. We need to investigate this point in future studies. Unusually, there was no relation between age and other variables. We thought this result was because the study subjects were mostly in their thirties.

In this research, the variables which affected Treg cell survival were male, CD4+ T cell and CD8+ T cell survival. Multiple regression (stepwise selection) was performed to determine the effect of CD4 survival, CD8 survival and sex on Treg survival and sex and cd8 survival showed a significant effect on Treg surviva rate (p = .0001 <.01). In male (B = 0.07984), the higher the CD8 survival rate (B = .619), the higher the Treg survival rate.

IV. Discussion

Treg cells are CD4+ cells that express high level of CD 25 (the IL-2 receptor α-chain) and the transcription factor forkhead box protein-3 (FoxP3). These cells account for 5-10% of circulating CD4+ T cells, suppress autoreactive lymphocytes, control innate and adaptive immune responses, and maintain self-tolerance, therefore they have a central role in protecting an individual from autoimmunity.[1] FoxP3 is a key regulator of Treg cell development and function. It is regarded as a reliable and specific marker for Treg cell.[10] However, the detection of FoxP3 with flow cytometry is only possible through laborious cell permeabilization and nonregulatory T cells can also express the intracellular marker FoxP3.[11] It has been recently recognized that Treg cells are expressing the IL-7 receptor α -chain (CD127) in significantly lower density and Treg function was inversely correlated with CD127 expression in human and mouse model of psoriasis. It was proved experimentally that CD3+/CD4+/CD25high/CD127low/- marker are FoxP3+ [12]. For routine testing, CD25high/CD127low/- phenotype is enough specific and sensitive to identify Treg cells.

The eximer laser emits UVB at a single wavelength of 308nm and the mechanism of action of excimer laser is similar to the action of UVB.[13] The 308nm excimer laser induces DNA breakage, upregulation of the tumor suppressor gene p53, and subsequent reduction of proto-oncogene BCL-2, resulting in apoptosis in keratinocytes and T lymphocytes.[14] When compared with the NBUVB, the 308 nm laser has an four times increased induction rate of T-cell apoptosis due to high irradiance[9]. So we thought the eximer laser might induce the difference of apoptosis according to T cell subtypes in vitro more distinctly than nonlaser UVB. Previous study[5] showed

50-100mJ/cm^2 of NBUVB in vitro induced apoptosis of T cells with highly dose dependent increase, we made a decision to irradiate in vitro at a dose of 100mJ/cm^2 with excimer laser.

There are many controversial reults of NBUVB effects on T cell subsets, although main mechanism of NBUVB is T lymphocytes apoptosis. Kagen et al.[15] reported that a single 10 minimal erythema dose (MED) of the eximer laser demonstrated preferential apoptosis of CD4+ T cells over CD8+ T cells in psoriatic patients. Moftah et al.[16] reported NBUVB therapy induced the greater decrease of circulating CD4+CD25+Foxp3+ Treg cells compared with CD4+CD25- Th cells in all vitiligo patients. In vivo or in vitro experiments on particular diseases might only reflect the result limited to the disease-specific situation because T lymphocytes might have disease- induced cellular dysfunction or numeral problems. We used healthy peripheral blood and intended to confirm the survival rate of normal T lymphocytes to excimer laser induced apoptosis which would be the standard. Our results showed that among Treg cells, CD4+ and CD8+ T cells, Treg cells are most resistant and CD4+ T cells are most susceptable to the excimer laser induced apoptosis.(p<0.05)

In many studies, phototherapy readily induces increases in Treg levels and restores Treg function, although the underlying mechanisms of Treg induction remain unclear. Yamazaki et al.[7] reported In a mouse model, UVB exposure induced thymus-derived FoxP3+ Treg cells(tTreg) with Treg-specific CpG hypomethylation, which showed in situ proliferation, not influx from peripheral blood or lymphatics. We had a question where the proliferating Treg cells originated from. Our hypothesis was that Treg cells might be most resistant to UVB induced apoptosis among T cell subsets and the accumlation of surviving Treg cells after UVB therapy might result in the foundation of in situ proliferation. Recently it was proved that dermal dendritic

cells(DC) was essential to boost Treg cells.[17] After UVB irradiation, dermal CD11b-type Langerin- DCs upregulated surface CD86 expression and a set of genes associated with immunological tolerance to induce the expansion of Treg cells.[18] Some authors propose transforming growth factor β(TGF-β) induces FoxP3 gene expression and CD25+ conversion in CD4+CD25- naïve T and UVB irradiation induces TGF- β expression in normal human keratinocytes.[6] In addition, serum vitamin D level and other mediators such as nitric oxide may be important for the expansion of Treg population by UVB therapy.[19,20]

Treg cells play a key role in protecting an individual from autoimmunity. For most autoimmune disease females have a greater prevalence than males.[21] Although there are many causes for sexual difference such as pregnancy, sex hormones, genetic predisposition and epigenetics, the differences between male and female immune systems are also important. Males have immune suprression when compared to females.[22] In vitiligo, female patients showed significant decrease in FoxP3 expression in Treg cells compared with male patient.[23] Recently in India, FoxP3 rs 3761548 polymorphism (-3279 C>A) has been found with especially female vitiligo patients, affecting the FoxP3 expression which can alter Treg functionality.[24] Even in healthy people, there is a lower regulatory T cell percentage in females than in males.[25] In present study, there was no significance difference in FoxP3+ Treg cell between males and females. However the Treg survival rate to eximer laser irradiation was lower in females than males with stasitically significant difference (p<0.05), which might result in the increase of immune reactivity and contribute to female preponderance in autoimmune disease.

There are several limitations in our study. First, the age of participants so young at average about 35 years old that we couldn't show

any survival difference of T cell subsets according to age. In general, there is not the significant correlation between Treg levels and age.[26] Second, the cytokines after T cells apoptosis was not tested in our study, which help to clear the Treg cell expansion mechanism of UVB irradiation. For example, TNF downmodulates the function of CD4+CD25+Treg cells, and TGF-β induced FoxP3 expression.[27]

V. Conclusion

We suggest that Treg cells are more resistant than CD4+ T cells in inducing T cell apoptosis to excimer laser irradiation. It can explain the increment of the proportion of Treg cells after ultraviolet phototherapy. Although this study was limited to a small number of volunteers and a relative narrow age range, this is the first study to reveal the apoptotic resistance of the Treg cells by ultraviolet B radiation.

Furthermore, it needs to be investigated the mechanism of difference in susceptibility to apoptosis in future study

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Table 1. Mean and median by patient characteristics

	Mean±S.E	MIN	MAX	MEDIAN	percentile	
					25	75
AGE	34.57±1.68	23	52	34	29	40
FoxP3%	3.51±.31	1.36	7.28	3.3	2.5	4.42
CD4 survival %	0.58±.03	0.26	0.85	0.58	0.47	0.69
Treg survival %	0.71±.03	0.3	0.96	0.72	0.63	0.8
CD4/Treg ratio	17.30±2.65	-14	38	18	12	26
CD8 survival %	0.64±.03	0.31	0.95	0.659	0.56	0.76
CD4/CD8 ratio	8.30±2.65	-27	27	12	0	16

Table 2. Multiple Paired t-test of the survival rate of Treg, CD4+ and CD8+ cells

Variables	Mean	Std. Error	95% CI	t	p
TREG survival	0.7066	0.02983	0.6447 - 0.7684		
CD4 survival	0.5833	0.03028	0.5206- 0.6461	-5.098 (1)	<0.0001
CD8 survival	0.6434	0.03381	0.5733 - 0.7135	-2.886 (2)	0.0086
CD4 survival /CD8 survival				3.762 (3)	0.0012

1) result for paired t-test between Treg and CD4: ($p<0.0001$)

2) result for paired t-test between Treg and CD8: ($p=<0.0086<0.0167$)

3) result for paired t-test between CD4 and CD8 : ($p=<0.0012<0.0167$)

Table 3. Independent T- Test the survival rate of Treg, CD4+ and CD8+ cells

Variable	Mean±S.E.M		t	p-value
	Male (N=11)	Female (N=12)		
Age	34.362.15	34.752.65	0.112	0.912
FoxP3%	3.810.51	3.240.37	-0.919	0.368
CD4 survival%	0.640.04	0.530.04	-1.890	0.073
Treg survival%	0.770.03	0.650.04	-2.335	0.030*
CD4/Treg ratio	16.274.99	18.25	0.356	0.719
CD8 survival	0.680.04	0.610.05	-1.137	0.268
CD4/CD8 ratio	4.554.72	11.752.47	1.352	0.196

The results of the Treg survival rate test by sex showed that the t statistic value was -2.335 and the p-value was less than the significance level 0.05 (5%) (p = 0.030). That is, Treg survival rate was higher in males than females.

Table 4. Spearman's correlation result of subjects(Rs(p-value))

variables	AGE	FoxP3%	CD4 survival	Treg survival	CD8 survival	CD4/Treg %	CD4/CD8 %
AGE	1.000						
FoxP3%	0.288 (0.182)	1.000					
CD4 survival	0.159 (0.469)	-0.094 (0.670)	1.000				
Treg survival	0.108 (0.624)	-0.248 (0.254)	0.728*** (0.000)	1.000			
CD8 survival	0.217 (0.321)	-0.027 (0.904)	0.877*** (0.000)	0.760*** (0.000)	1.000		
CD4/Treg %	-0.163 (0.458)	-0.050 (0.822)	-0.567** (0.005)	0.074 (0.738)	-0.354 (0.097)	1.000	
CD4/CD8 %	-.101 (0.647)	-.072 (0.743)	-.252 (0.247)	-.024 (0.913)	.149 (0.497)	.258 (0.235)	1.000

*Rs is spearman's correlation coefficient

There was a positive correlation between CD4 and Treg (ρ = 0.728, p <0.01) and a positive correlation between CD4 and CD8 (ρ = 0.877, p <0.01) and a negative correlation between CD4 and CD4 / Treg (P = -0.567, p = 0.005, p <0.05). There was a positive correlation between CD8 and Treg (ρ = 0.760, p <0.01) and no correlation between CD8 and CD4 / Treg (ρ = -0.357, p = 0.097> 0.05).

Table 5. Multiple regression analysis on Treg survival

	B	SE	T	p
(constant)	0.2701			
sex	0.07984	0.0370	2.153	0.0437
CD8 survival	0.6191	0.1168	5.301	<0.0001
	R^2= .676 F = 13.195 p = .0001			

Among sex, CD4 survival and CD8 survival, sex and cd8 survival showed a significant effect on Treg surviva rate (p = .0001 <.01). In male(B = 0.07984), the higher the CD8 survival rate (B = .619), the higher the Treg survival rate.

Figure 1. Box - and- Whisker diagram of the survival rate of Treg, CD4+ and CD8+ cells. Treg survival was the highest, followed by CD8 survival and CD4 survival

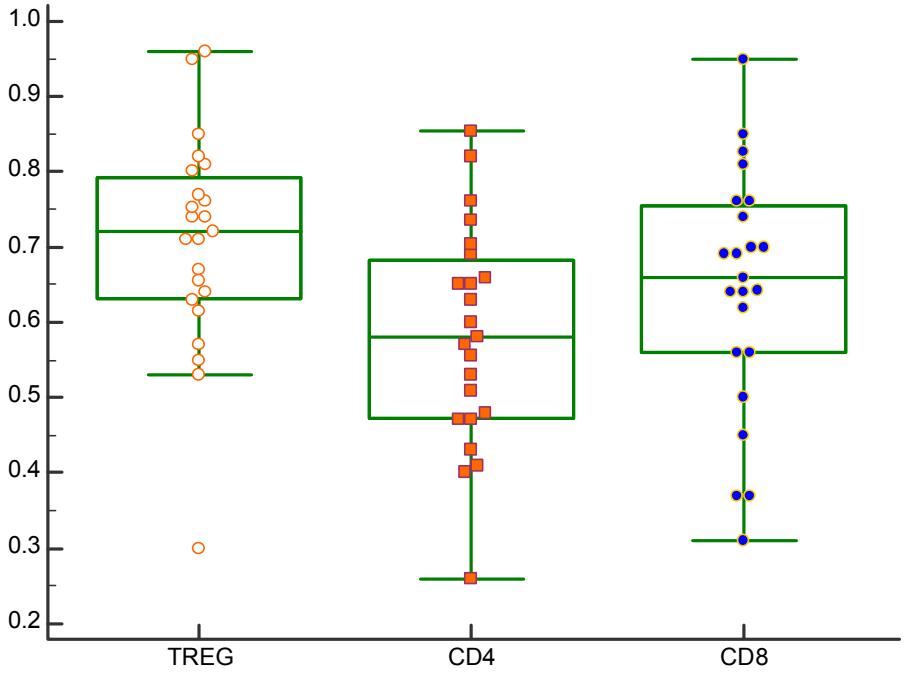
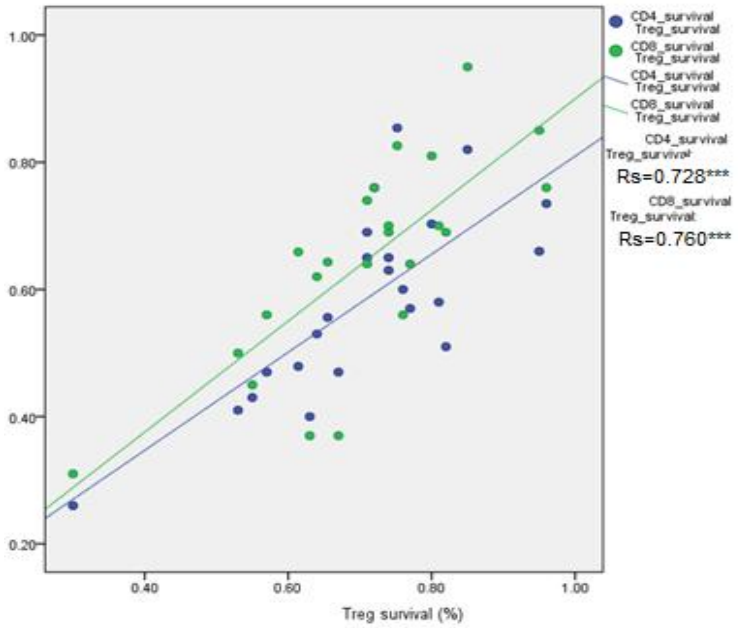
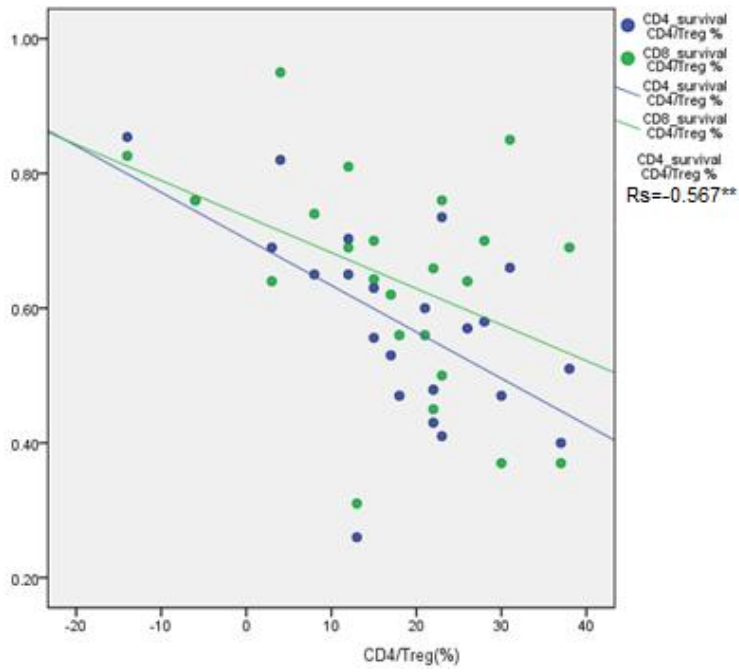


Figure 2. Nonparametric Correlation between variables in patients.

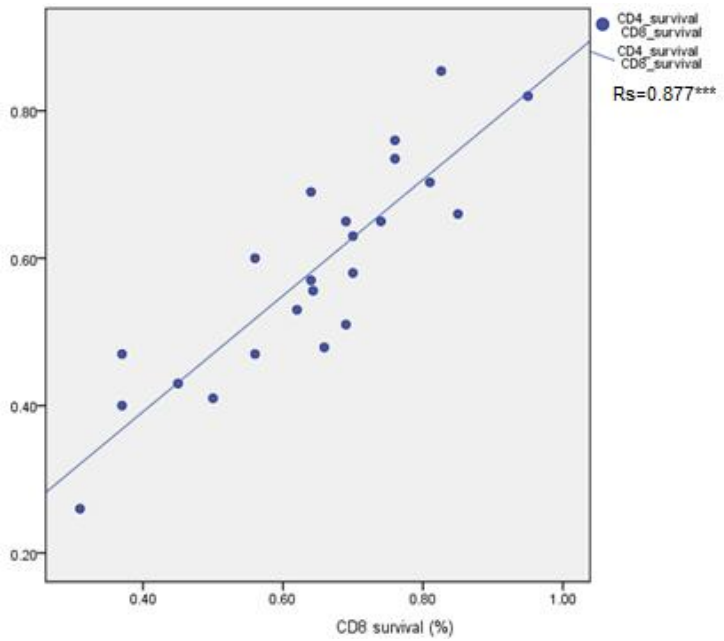
A)



B)



C)



(A) A positive correlation between CD4 survival AND Treg survival was found in the patients (Rs=0.728, P=0.000). Also a positive correlation between CD8 survival and Treg survival was found in the patients (Rs=0.760, p=0.000).

(B) A negative correlation between CD4 survival and CD4/Treg was found in the patients (Rs=-0.567** , p=0.005)). No statistically significant correlation between CD8_survival and CD4/Treg was found.

(C) Also a positive correlation between CD4 survival and CD8 survival was found in the patients (Rs=0.877, p=0.000).