Global Scientific JOURNALS

GSJ: Volume 8, Issue 6, June 2020, Online: ISSN 2320-9186
www.globalscientificjournal.com

Interleukin-23 and Interleukin-25 activities following recombinant human Interleukin-2 administration in an experimental model of Streptozotocin-induced diabetes mellitus

Abu El-Nazar S., Ghoneim H., Abou-Shamaa L., Shalaby T., Merssal B., Abdelfattah Z., Elbigawy N.

KeyWords

Interleukin-2, Interleukin-23, Interleukin-25, Type1 diabetes.

Abstract:

Type1 diabetes (T1D) is a complex chronic autoimmune inflammatory disease in which induced cytokine production creates dynamic environments for evolution and activity of effector as well as regulatory cells. Low-dose IL-2 has been applied for treatment recent-onset T1D in NOD mice. Different IL-2 formulations, for instance nanoparticle encapsulation, have been implicated to improve the therapeutic utility of low-dose IL-2 to achieve precise suppression of pathological immune responses. So, the current study intended to evaluate the effect of ultra-low-dose rhIL-2 therapy on the inflammatory process in multiple low dose streptozotocin (MLD STZ) induced T1D as well as the influence of chitosan (CS) nanosphere encapsulation on the proposed effect. Male inbreed Balb/c mice were divided into 5 groups; **Normal** (Normal mice), **T1D**: (T1D induced by MLD-STZ), **T1D (Nano)**: T1D mice treated with free CS-TPP-NPs, **T1D (rIL-2)**: T1D mice treated with free rhIL-2, and **T1D (rIL-2/Nano)**: T1D mice treated with rhIL-2 encapsulated into CS-TPP-NPs. Levels of IL-23 and IL-25 (for inflammatory and anti-inflammatory arms, respectively) were assessed in splenocytes culture supernatant. Results showed that both rhIL-2 and rhIL-2/CSNPs significantly decreased IL-23 as compared to corresponding group in T1D with maximum decrease at day 21 post treatment. Both rhIL-2 and rhIL-2/CSNPs significantly decreased IL-25 at days 7 and 14. All through the three time intervals, IL-25 remained significantly higher in rhIL-2/ CSNPs than in rhIL-2. In conclusion, Ultra-low dose rhIL-2 ameliorated the diabetic state at day 7. rhIL-2/CSNP is more prone to induce an anti-inflammatory stat. The anti-diabetic effect of rhIL-2 or rhIL-2/CS-NPs is not directly dependent on alterations in IL-23 and IL-25 production.

© GSJ

Introduction:

Type 1 diabetes (T1D) is a classical example of organ-specific inflammatory autoimmune disorders initiated by misrecognition of β cell autoantigens in pancreatic islets of Langerhans leading to insulitis followed by β cell destruction. Notably, a complex panel of cytokines is believed to be actively involved in the induction, perpetuation or control of insulitis (Clark etal., 2017). Among these cytokines, Interleukin-23, primarily secreted by tissue resident macrophages and DCs, amplifies and sustain the proliferation of pathogenic Th17 which initiates production of other pro-inflammatory mediators such as IL-1, TNFα, IL-6, IL-8, CCL20 and G-CSF (Duvallet et al., 2011). Interleukin-25 (IL-17E), on the other hand, is constitutively expressed by a variety of cell populations including polarized Th2, $CD8^+$ T cells, macrophages, DCs, mast cells and eosinophils as well as epithelial and endothelial cells. Interestingly, the function of this cytokine is linked to its ability to amplify Th2/Th9-immunity and to suppress Th1/Th17 cell responses; thus promoting allergic responses and attenuating detrimental inflammation associated with human autoimmune disorders (Iwakura et al., 2011).

Streptozotocin (STZ), is an antibiotic produced by the bacterium *Streptomyces achromogens* and is widely applied, among several other approaches, for creating an experimental model for diabetes mellitus in rodents (Wu et al., 2015).

Interleukin-2 is a multifunctional cytokine produced mainly by stimulated $CD4^+$ T cells and by $CD8^+$ T cells, NKT cells, activated DCs and mast cells. It is essential for the differentiation, expansion and stability of Treg cells involved in immune suppression and self-tolerance. Meanwhile, it also stimulates Teff cells to promote inflammatory immune responses. It is postulated that Teff cells are sustained in response to high levels of IL-2 while Treg cells are selectively maintained in response to persistent low doses of Il-2. Subsequently, IL-2 has been determined as an immunotherapeutic agent in cancer as well as autoimmune diseases. In the last few decades, multiple approaches are developed to preferentially target IL-2 towards Treg (Abbas et al., 2018).

Chitosan is a biocompatible, biodegradable and nontoxic polymer derived from chitin (the main component of the exoskeleton of arthropods) by partial de-acetylation. Chitosan nano-particles are FDA approved as a drug delivery system to increase the half life of essential drugs by controlling the rate of their release (Mohammed et al., 2017). The present study aimed at evaluating the effect of low dose recombinant human IL-2 loaded

into chitosan nanosphere on the interplay between IL-25 and IL-23 released by splenocytes obtained from STZ-induced T1D in male inbreed Balb/c mice.

Material and methods

Induction of Diabetes

Male inbreed Balb/c mice represented the experimental animal model of the present study. Animal handling and manipulation were carried out in accordance with the ethical guidelines of the Medical Research Institute, University of Alexandria (Appendix I. Guiding principles for biomedical Research involving animals, 2011).

Diabetes mellitus was induced in male Balb/c mice by a multiple low dose protocol adopted from and composed of 5 consecutive intraperitoneal injections with streptozotocin (Sigma, USA) at a dose of 50 mg/kg body weight.

Monitoring of diabetes

For diabetes follow-up, glycaemia was monitored by ACCU-CHECK glucometer (Roche®, Swiss). Diabetes onset was defined by hyperglycaemia exceeding 200 mg/dl. Fasting insulin levels were also quantified by Cobas® Insulin electro-chemiluminescence immunoassay "ECLIA" following animal scarification.

Preparation of free and IL-2-loaded chitosan nanosphere

Free chitosan-Tri-poly-phosphate nanospheres (Sigma, USA) were prepared using the ionic gelation method according to Mohammadpour Dounighi N, et al., 2012. Briefly, Penta-basic sodium tripolyphosphate (TPP) aqueous solution (0.1% w/v) was added drop wise into chitosan solution (0.1% w/v acetic acid solution) in a ratio of 2.5:1. This mixture was stirred at room temperature at 500 rpm for 5 min. The prepared nanospheres were separated by centrifugation at 20,000 rpm for 30 minutes. Supernatants were decanted and pellets were re-suspended in physiological saline.

For preparation of cytokine-loaded chitosan nanosphere, 2000 IU of recombinant human IL-2 in a total volume of 12.5 µl (Proleukin, Biolegend, USA) were added to the prepared TPP solution before drop wise addition and stirring with chitosan solution. For separation of chitosan IL-2 nanospheres, the mixture was centrifuged at 20,000 rpm for 30 minutes followed by supernatant decantation and re-suspension in saline.

Characterization of chitosan nanoparticles

The IL-2 loading capacity was measured using ultraviolet spectrophotometer at 595 nm. The encapsulation efficiency% was calculated according to the following equation (Mohammadpour Dounighi N et al., 2012):

$$\text{Encapsulation efficiency } \% = \frac{T_p - T_f}{T_p} \times 100$$

Where Tp is the total amount of IL-2 and Tf is the free IL-2 in the supernatant (Mohammadpour Dounighi N et al., 2012).

Topography of the prepared nanoparticles was defined by Scanning Electron Microscope (Quanta 400 ESEM/EDAX ®, 20 kV, Netherlands), bond type structure and functional groups was monitored by Fourier Transforms Infrared Spectroscopy (Shimadzu, FT-IR-8400S, Japan) while particle homogeneity and dispersity were estimated using a Zetasizer (Malvern Instruments, UK).

© GSJ

Recombinant human IL-2 treatment

Diabetic mice were treated with free IL-2 by 5 consecutive intraperitoneal daily injections (20 IU each) of rhIL-2. IL-2 loaded chitosan nanospheres were administered to diabetic mice as a single dose of 100 IU/mouse by the same route.

Animal scarification

Mice from different groups were scarificed by cervical dislocation at various time durations (day 5, day 14 and day 21 after the last dose of IL-2).

Splenocyte isolation and cell culture assays

Immediately following mice scarification, spleens were dissected out and mashed by the plunger of sterile syringes to obtain sufficient amounts of splenocytes that were counted and tested for viability by Trypan blue dye exclusion. Splenocytes were suspended in complete culture medium composed of RPMI-1640 (Sigma, USA) supplemented with heat-inactivated fetal calf serum (Sigma, USA, 10%), penicillin (100 U/ml, Sigma, USA), streptomycin (100 U/ml, Sigma, USA) and gentamycin (20 g/ml, Sigma, USA).

Splenocytes were maintained in a short term cultures (48 hr) in a humidified CO2 incubator at an atmosphere of 37℃ and 5% CO2 (Thermo ™, USA) either in the presence or absence of the polyclonal splenocyte activator concanavalin A (final dilution of 10 ug/ml). The culture supernatants were harvested at the end of the culture period, centrifuged at 2000 rpm and aliquots were maintained at -70 ºC until use in cytokine assays.

Quantification of IL-23 and IL-25

IL-23 and IL-25 levels were quantified in culture supernatants of splenocytes from all animal groups using sandwich enzyme-linked immune-sorbent assay (ELISA) using commercially available kits (cloud-clone®, USA).

Results

Characterization of free versus rhIL-2 loaded chitosan nanoparticles

The IL-2 loading and encapsulation efficiency of CS-TPP-NPs was estimated from the mean of 6 different preparations to be reproducible ranging around 95-97%. The SEM images revealed spherical porous morphology with average diameter of 91.8 and 276.3 nm for free versus rhIL-2/CS-TPP-NPs, respectively, figuring ~ 300% increase in average diameter after loading with rhIL-2.

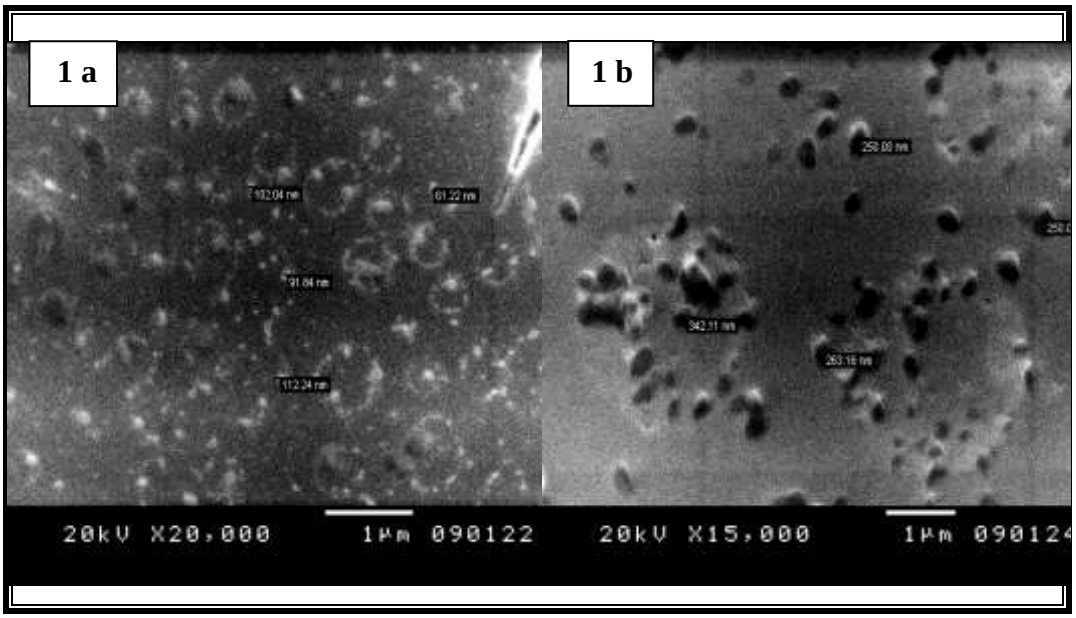

Figure (1): SEM images for CS-TPP-NPs as (a) compared to their relevant rhIL-2/ CS-TPP-NPs (b).

The PDI of free and rhIL-2/CS-TPP-NPs were 0.269 versus 0.328 indicating homogenous dispersion and greater particle stability while zeta potential was 13.7 versus 24.2 mv indicating augmented charge due to rhIL-2 loading.

Table (1): Zetasizer measurements

	Zeta potential	PDI
CS-TPP-NPs	**13.7± 3.04**	**0.269**
rhIL-2/ CS-TPP-NPs	**24.2± 3.36**	**0.328**

The FTIR spectra curves corresponding to the characteristic functional groups of free and rhIL-2/CS-TPP-NPs were almost identical indicating no change in characters of CS-TPP-NPs following loading with rhIL-2.

Table (2): The characteristic spectra corresponding to characteristic functional groups of CS-TPP-NPs vs rhIL-2/ CS-TPP-NPs

Functional groups	Characteristic spectra (/cm)	
	CS-TPP-NPs	**rIL-2/Nano**
Hydroxyl (OH) and amino (NH2) groups	3335.60	3342.5
Alkane(C-H)	2089.05	2090.30
Carbonyl group (C=O) of acetyl groups	1642.50	1643
Saccharide structure	542	583.50

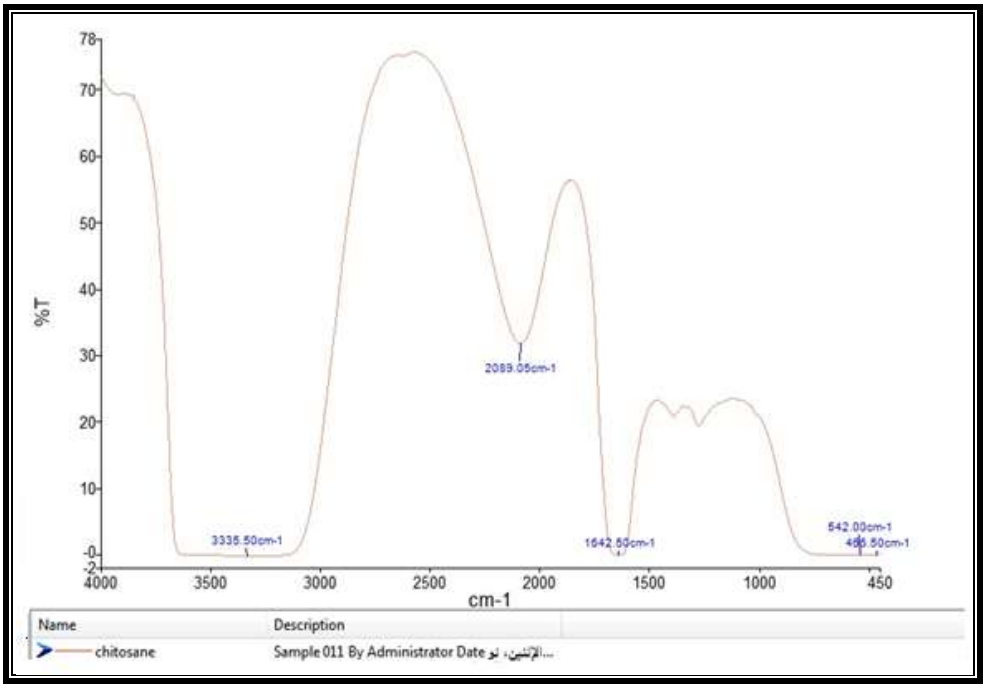

Figure (2): FT-IR specrum of CS-TPP-NPs.

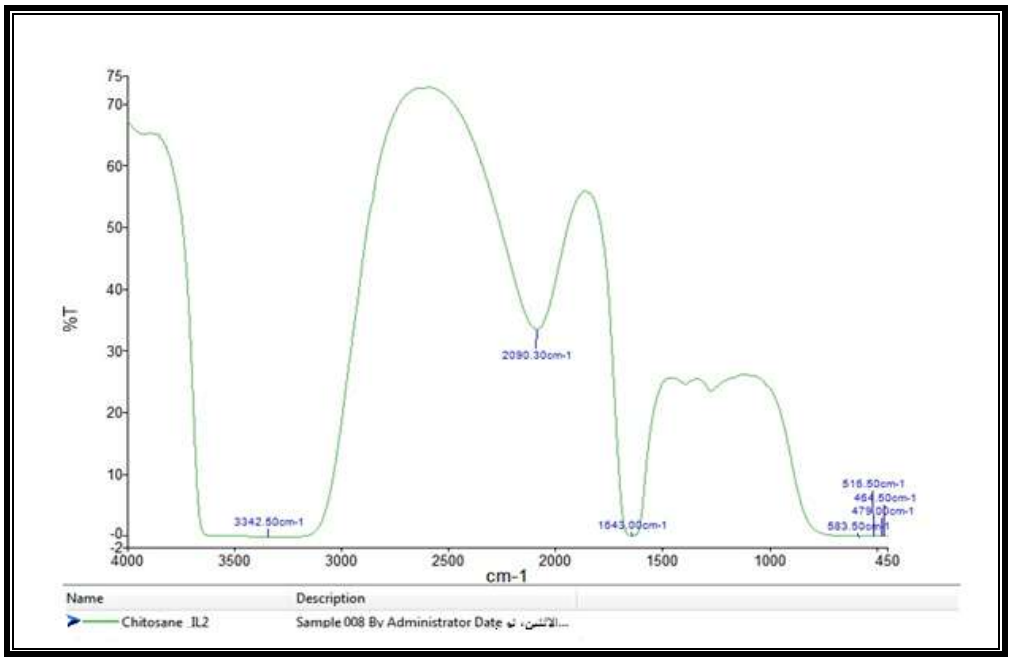

Figure (3): FT-IR specrum of CS-TPP-NPs loaded with rIL-2.

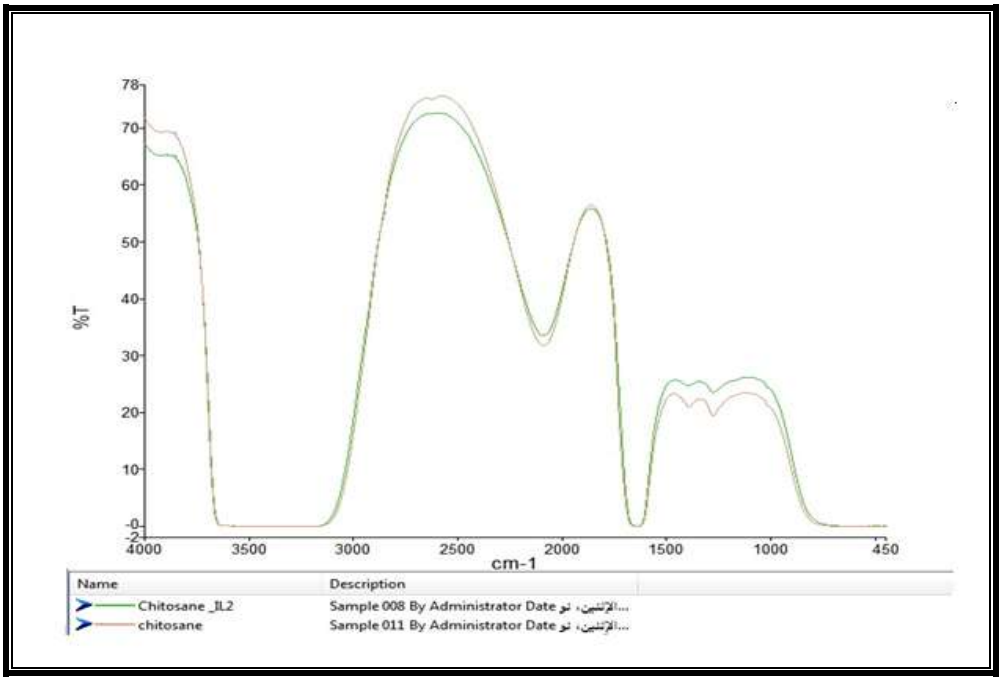

Figure (4): FT-IR specrum of free CS-TPP-NPs vs rIL-2/CS-TPP-NPs.

© GSJ

Effect of rhIL-2 treatment on STZ-induced diabetic status

One week after rhIL-2 treatment, glucose levels were significantly dropped to almost normal levels. This was coincident with selectively ameliorated insulin secretion only but diabetic mice given free IL-2.

Table (3): Statistical analysis of blood glucose (mg/dl) and serum insulin (U/L) results in diabetic mice one week after treatment with free or chitosan-loaded rhIL-2 as compared to diabetic un-treated and normal mice

Parameter	Normal (n-10)	T1D (n-5)	T1D/IL-2 (n-10)	T1D/Ch+IL-2 (n-10)
Glucose (mg/dl)				
Range	86-120	242-560	70-140	66-148
Mean	101.1	357.2	99.4	96.1
SD	12.11473	136.468	22.052	29.794
P		0.011*	0.834	0.632
p1			0.011*	0.01*
p2				0.8
Insulin (U/L)				
Range	1.02-1.77	0.86-1.05	1.03-1.53	0.97-1.31
Mean	1.337	0.958	1.195	1.11
SD	0.302	0.077	0.141	0.12
P		0.003*	0.202	0.05*
p1			0.002*	0.02*
p2				0.2

p, p1 and p2 value: Significant variation at $p \leq 0.05$

Two weeks after rhIL-2, glucose levels were still significantly reduced due to treatment although higher than normal mice. Insulin secretion was retained almost normal but only in diabetic mice given free IL-2.

Table (4): Statistical analysis of blood glucose (mg/dl) and serum insulin (U/L) results in diabetic mice 2 weeks after treatment with free or chitosan-loaded rhIL-2 as compared to diabetic un-treated and normal mice

Parameter	Normal (n-10)	T1D (n-5)	T1D/IL-2 (n-10)	T1D/Ch+IL-2 (n-10)
Glucose (mg/dL)				
Range	86-120	253-500	164-283	206-366
Mean	101.1	378.6	213.800	292.4
SD	12.11473	103.5243933	37.935	51.64
P		0.004*	<0.0001*	<0.0001*
p1			0.02*	0.14
p2				0.001*
Insulin (U/L)				
Range	1.02-1.77	0.94-1.02	1.14-1.42	0.87-1.14
Mean	1.337	0.986	1.278	0.979
SD	0.302	0.030	0.099	0.085
P		0.005*	0.57	0.005*
p1			<0.0001*	0.82
p2				<0.0001*

p, p1 and p2 value: Significant variation at $p \leq 0.05$

By the end of the study, glucose levels were dramatically reduced in mice given chitosan loaded IL-2 not the free form. Surprisingly, this wasn't consistent with insulin results which escaped the normal counter regulatory characteristic.

Table (5): Statistical analysis of blood glucose (mg/dl) and serum insulin (U/L) results in diabetic mice 3 weeks after treatment with free or chitosan-loaded rhIL-2 as compared to diabetic un-treated and normal mice

Parameter	Normal (n-10)	T1D (n-5)	T1D/IL-2 (n-10)	T1D/Ch+IL-2 (n-10)
Glucose (mg/dL)				
Range	86-120	246-560	171-491	128-202
Mean	101.1	366.800	319.4	161.9
SD	12.11473	128.295	110.324	26.635
P		0.01*	0.0001*	<0.000.1*
p1			0.50	0.02*
p2				0.001*
Insulin (U/L)				
Range	1.02-1.77	0.86-1.04	0.99-1.2	0.79-0.95
Mean	1.337	0.952	1.121	0.912
SD	0.302	0.078	0.070	0.047*
P		0.003*	0.05*	0.002
p1			0.004*	0.337
p2				<0.0001*

p, p1 and p2 value: Significant variation at $p \leq 0.05$

© GSJ

Effect of T1D induction and rhIL-2 treatment on IL-23 and IL-25

One week following rhIL-2, the IL-23 levels that were elevated due to T1D induction were almost normalized. In contrast, IL-25 that wasn't changed due to T1D induction, showed significant reduction due to treatment.

Table (6): Statistical analysis of IL-23 and IL-25 (ng/L) results in splenocyte culture supernatants collected from diabetic mice one week after treatment with free or chitosan-loaded rhIL-2 as compared to diabetic un-treated and normal mice

Parameter	Normal (n-10)	T1D (n-5)	T1D/IL-2 (n-10)	T1D/Ch+IL-2 (n-10)
IL-23 (ng/L)				
Range	2.919-3.597	3.832-4.273	3.152-3.774	3.201-3.674
Mean	3.192	4.012	3.425	3.393
SD	0.338	0.198	0.237	0.181
P		<0.0001*	0.1	0.12
p1			0.0004*	0.0003*
p2				0.738
IL-25 (ng/L)				
Range	2.094-2.306	1.611-2.279	0.93-1.329	1.001-2.051
Mean	2.198	1.997	1.129	1.657
SD	0.091	0.319	0.138	0.377
P		0.275	<0.0001*	0.0013*
p1			0.004*	0.121
p2				0.0015*

p, p1 and p2 value: Significant variation at $p \leq 0.05$

© GSJ

Two weeks following rhIL-2, there was a significant reduction in IL-23 due to treatment relative to diabetic mice that were still significantly higher than normal. Frank reduction in IL-25 was recorded in treated mice although diabetic untreated group were almost normal.

Table (7): Statistical analysis of IL-23 and IL-25 (ng/L) results in splenocyte culture supernatants collected from diabetic mice 2 weeks after treatment with free or chitosan-loaded rhIL-2 as compared to diabetic un-treated and normal mice

Parameter	Normal (n-10)	T1D (n-5)	T1D/IL-2 (n-10)	T1D/Ch+IL-2 (n-10)
IL-23 (ng/L)				
Range	2.919-3.597	3.88-4.273	3.274-4	3.414-3.72
Mean	3.192	3.959	3.547	3.579
SD	0.338	0.176	0.267	0.123
P		<0.0001*	0.02*	0.006*
p1			0.004*	0.005*
p2				0.736
IL-25 (ng/L)				
Range	2.094-2.306	2.009-2.279	0.774-2.037	1.441-1.881
Mean	2.198	2.171	1.384	1.748
SD	0.091	0.148	0.445	0.168
P		0.720	0.0002*	<0.0001*
p1			0.0003*	0.001*
p2				0.033*

p, p1 and p2 value: Significant variation at $p \leq 0.05$

Three weeks after rhIL-2 treatment, a highly significant reduction in IL-23 was recorded in treated groups relative to the diabetic untreated mice that were retained higher than normal. A significant decrease in IL-25 was recorded in treated groups as compared to both untreated and negative control mice, pronounced in mice given free rather than chitosan loaded IL-2.

Table (8): Statistical analysis of IL-23 and IL-25 (ng/L) results in splenocyte culture supernatants collected from diabetic mice 3 weeks after treatment with free or chitosan-loaded rhIL-2 as compared to diabetic un-treated and normal mice.

Parameter	Normal (n-10)	T1D (n-5)	T1D/IL-2 (n-10)	T1D/Ch+IL-2 (n-10)
IL-23 (ng/L)				
Range	2.919-3.597	3.832-4.273	2.568-2.968	2.857-3.367
Mean	3.192	3.986	2.788	3.008
SD	0.338	0.183	0.158	0.198
P		<0.0001*	0.005*	0.160
p1			<0.0001*	<0.0001*
p2				0.014*
IL-25 (ng/L)				
Range	2.094-2.306	1.611-2.279	0.532-1.611	0.640-2.591
Mean	2.198	2.084	1.092	1.525
SD	0.091	0.287	0.410	0.880
P		0.431	<0.0001*	0.04*
p1			0.0002*	0.1
p2				0.2

p, p1 and p2 value: Significant variation at $p \leq 0.05$

Correlations in non-diabetic mice

Significant positive correlations in **normal** mice were recorded between glucose and IL-23 as well as between insulin and IL-25 (Figures 5-6). Significant negative correlations were recorded between glucose with insulin in one hand and IL-25 on the other hand as well as between IL-23 with insulin on one hand and IL-25 on the other hand (Figures 7-8).

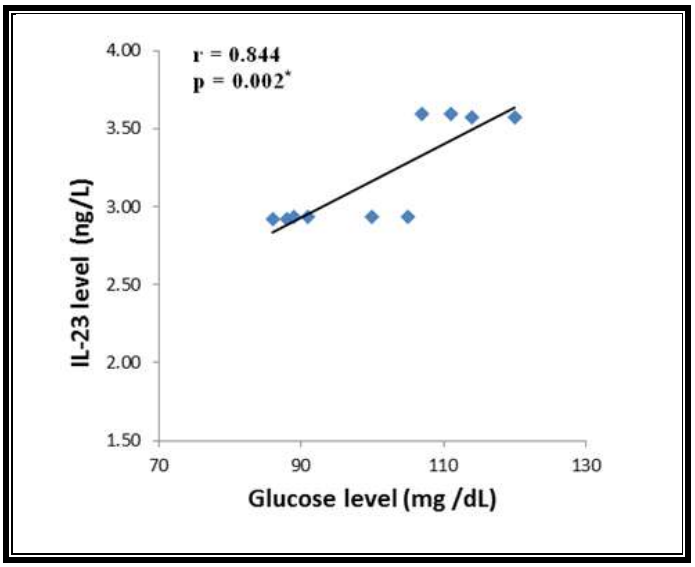

Figure (5): Correlation between glucose and IL-23 levels in Normal group.

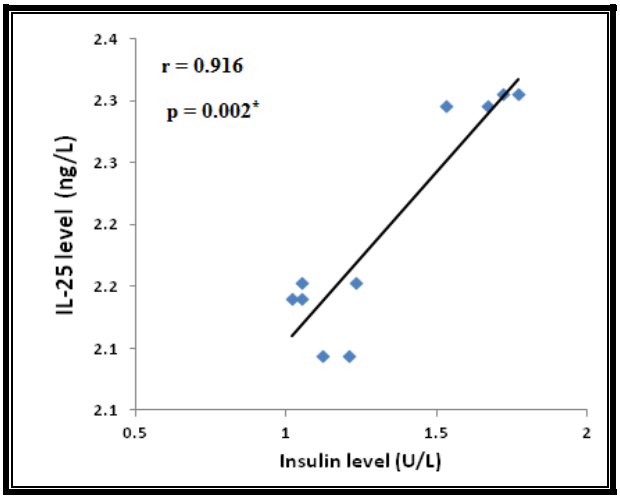

Figure (6): Correlation between insulin and IL-25 levels in Normal group.

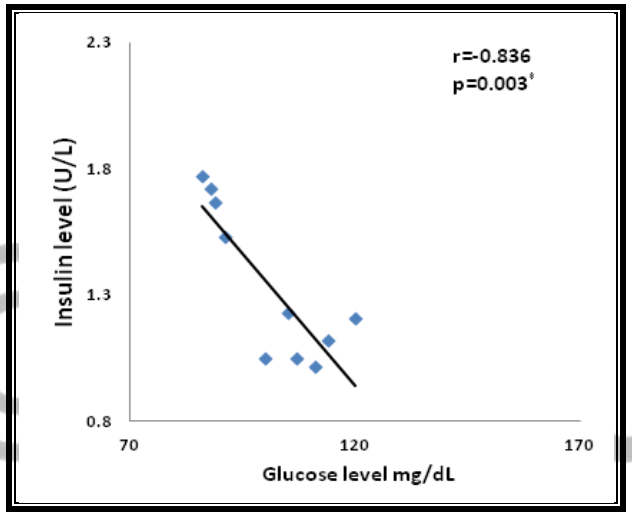

Figure (7): Correlation between insulin and glucose levels in Normal group.

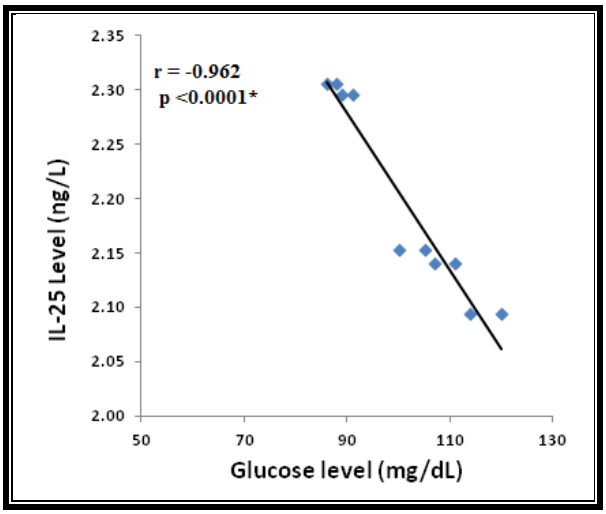

Figure (8): Correlation between glucose and IL-25 levels in Normal group.

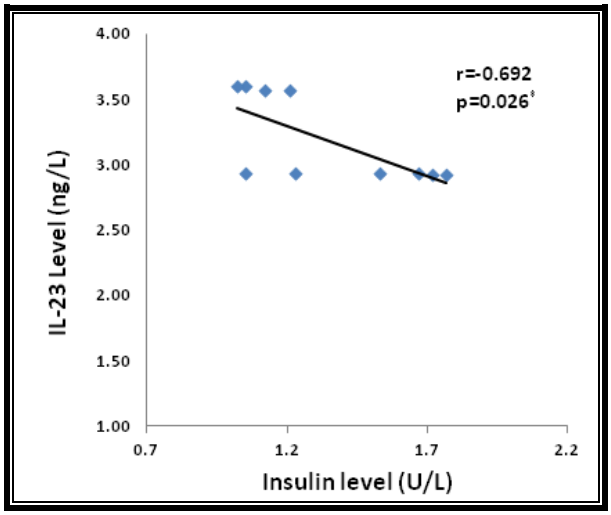

Figure (9): Correlation between insulin and IL-23 levels in Normal group.

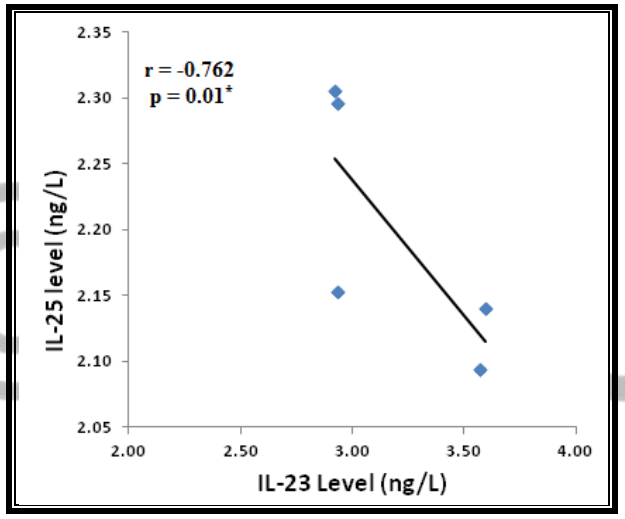

Figure (10): Correlation between IL-25 and IL-23 levels in Normal group.

Correlations in diabetic mice

Significant positive correlations were restricted to glucose with IL-23 (Figure 11). On the other hand, significant negative correlations were recorded between insulin with glucose and IL-23 (Figures 12-13).

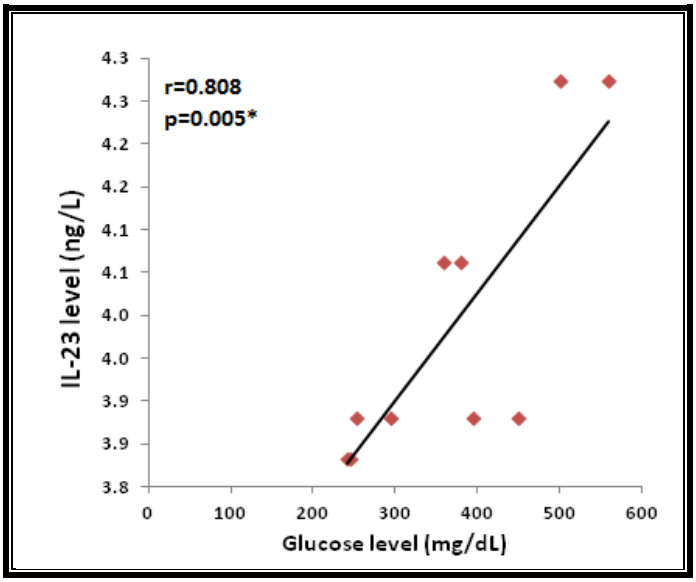

Figure (11): Correlation between glucose and IL-23 levels in T1D group.

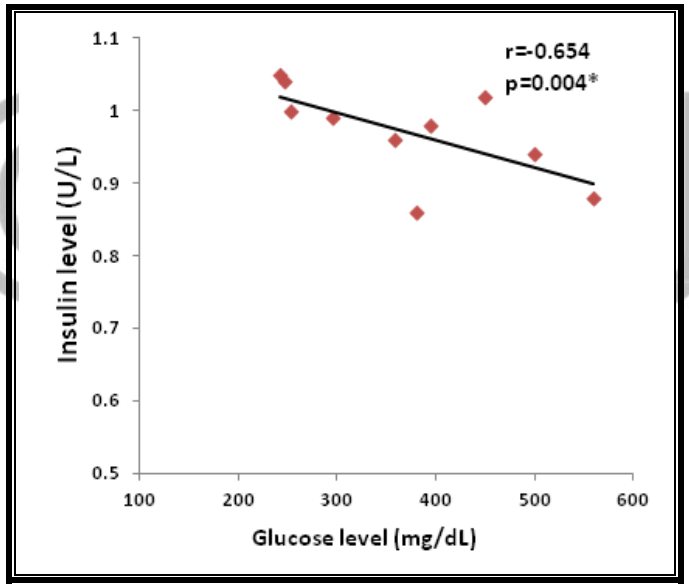

Figure (12): Correlation between insulin and glucose levels in T1D group.

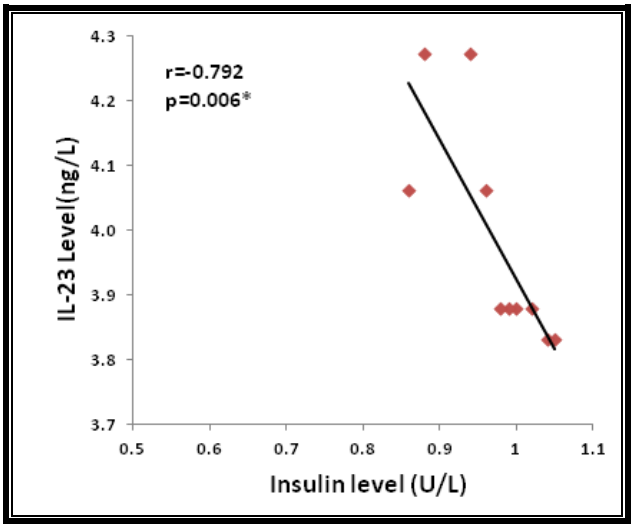

Figure (13): Correlation between insulin and IL-23 levels in T1D group.

Time variation of assessed parameters through 21 days

Both free IL-2 and IL-2 loaded CS-NP decreased the mean blood glucose level along the 21 days post treatment with a maximum significant decrease at day 7.

Table (9): Variation of glucose level through 21 days post treatment

Group	Glucose (Mean±SD mg/dL)			P1	P2	P3
	7 days	**14 days**	**21 days**			
T1D/rIL-2						
Mean±	99.4±	213.8±	319.4±	<0.0001*	0.02*	<0.0001*
SD	22.05	37.935	110.324			
T1D/rIL-2/Nano						
Mean±	96.1±	292.4±	162±	<0.0001*	<0.0001*	<0.0001*
SD	29.79	51.64	26.635			

N.B. The mean ± SD of glucose level in untreated T1D group= 368±111.205
P: Level of significance
*P: significant difference at p≤0.05.
P1: difference between 7 days and 14 days.
P2: difference between 14 days and 21 days.
P3: difference between 7 days and 21 days.

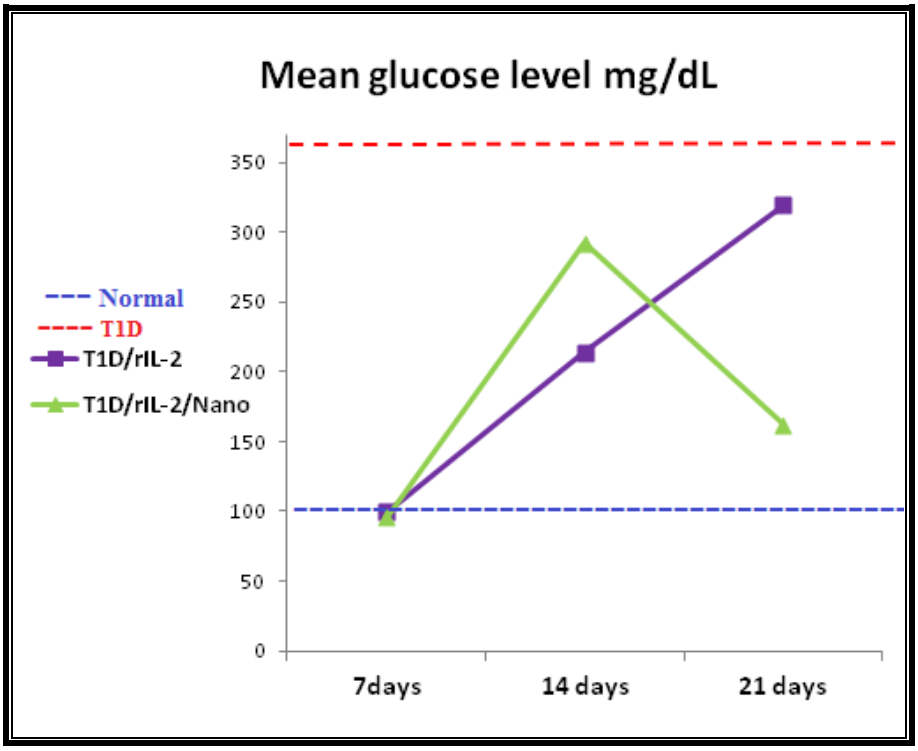

Figure (14): Time variation of glucose level through 21 days in T1D/rIL-2 and T1D/rIL-2/Nano groups.

Table (10): Variation of insulin level through 21 days post treatment

Group	Insulin (Mean±SD U/L)			P1	P2	P3
	7 days	**14 days**	**21 days**			
T1D/rIL-2						
Mean±	1.195±	1.278±	1.121±	0.148	0.001*	0.16
SD	0.14	0.099	0.07			
T1D/rIL-2/Nano						
Mean±	1.11±	0.979±	0.925±	0.001*	0.08	0.001*
SD	0.119	0.085	0.024			

N.B. The mean ± SD of insulin level in untreated T1D group= 0.972±0.062
P: Level of significance
*P: significant difference at p≤0.05.
P1: difference between 7 days and 14 days.
P2: difference between 14 days and 21 days.
P3: difference between 7 days and 21 days.

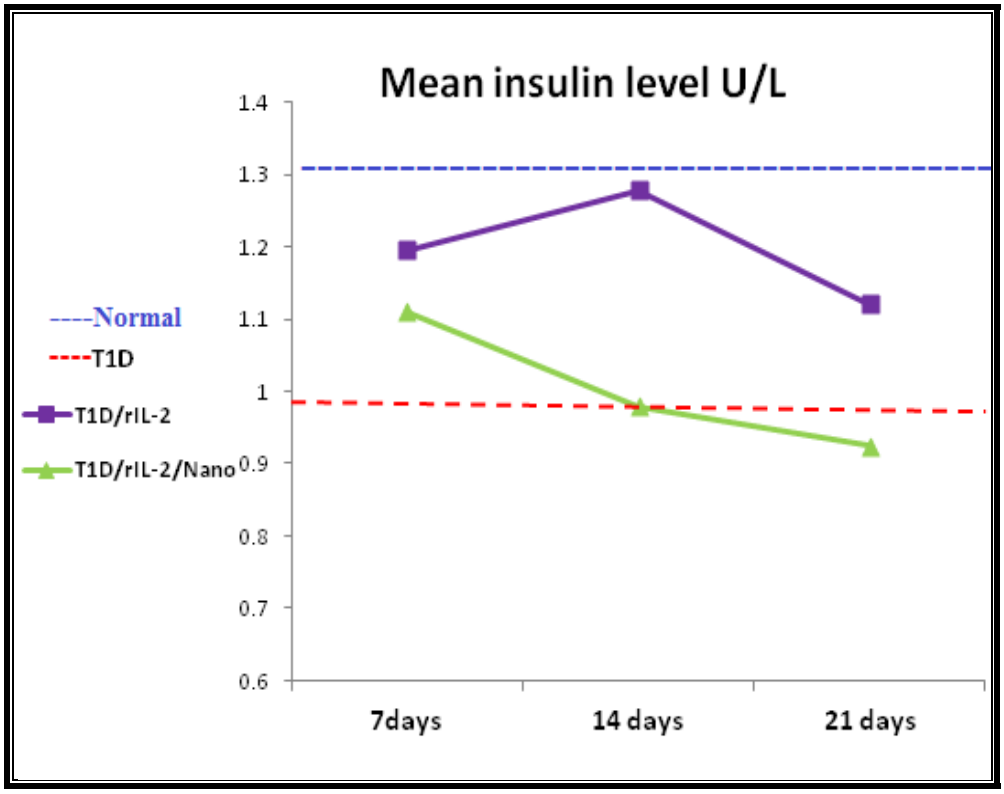

Figure (15): Time variation of insulin level through 21 days in T1D/rIL-2 and T1D/rIL-2/Nano groups.

Both free IL-2 and IL-2 loaded CS-NPs decreased the mean IL-23 level nearly to the same extent along the 21 days post treatment with maximum significant reduction at day 21.

Table (11): Variation of IL-23 through 21 days post treatment

Group	IL-23 (Mean±SD ng/L)			P1	P2	P3
	7 days	**14 days**	**21days**			
T1D/rIL-2						
Mean±	3.425±	3.547±	2.788±	0.3	<0.0001*	<0.0001*
SD	0.24	0.267	0.158			
T1D/rIL-2/Nano						
Mean±	3.393±	3.579±	3.008±	0.02*	<0.0001*	0.0003*
SD	0.181	0.123	0.198			

N.B. The mean ± SD of IL-23 level in untreated T1D group= 3.986 ± 0.173
P: Level of significance
*P: significant difference at p≤0.05.
P1: difference between 7 days and 14 days.
P2: difference between 14 days and 21 days.
P3: difference between 7 days and 21 days.

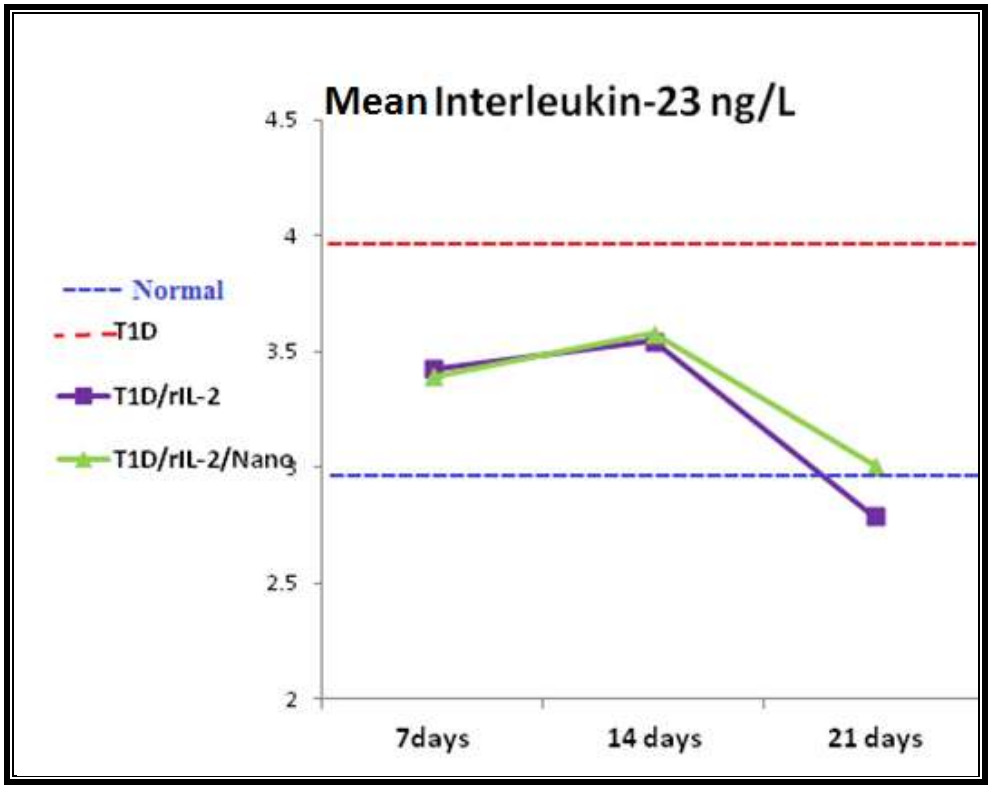

Figure (16): Time variation of IL-23 level through 21 days in T1D/rIL-2 and T1D/rIL-2/Nano groups.

Both free IL-2 and IL-2 loaded CS-NPs decreased the mean IL-25 level, however, the mean IL-25 level was significantly lower in case of free IL-2 along the time intervals.

Table (12): Variation of IL-25 through 21 days post treatment

Group	IL-25 (Mean ± SD ng/L)			P1	P2	P3
	7 days	**14 days**	**21 days**			
T1D/rIL-2						
Mean±	1.129±	1.384±	1.092±	0.112	0.144	0.791
SD	0.14	0.445	0.41			
T1D/rIL-2/Nano						
Mean±	1.657±	1.748±	1.525±	0.499	0.451	0.671
SD	0.377	0.168	0.88			

* N.B. The mean ± SD of IL-25 level in untreated T1D group= 2.084 ± 0.271
P: Level of significance
*P: significant difference at p≤0.05.
P1: difference between 7 days and 14 days.
P2: difference between 14 days and 21 days.
P3: difference between 7 days and 21 days.

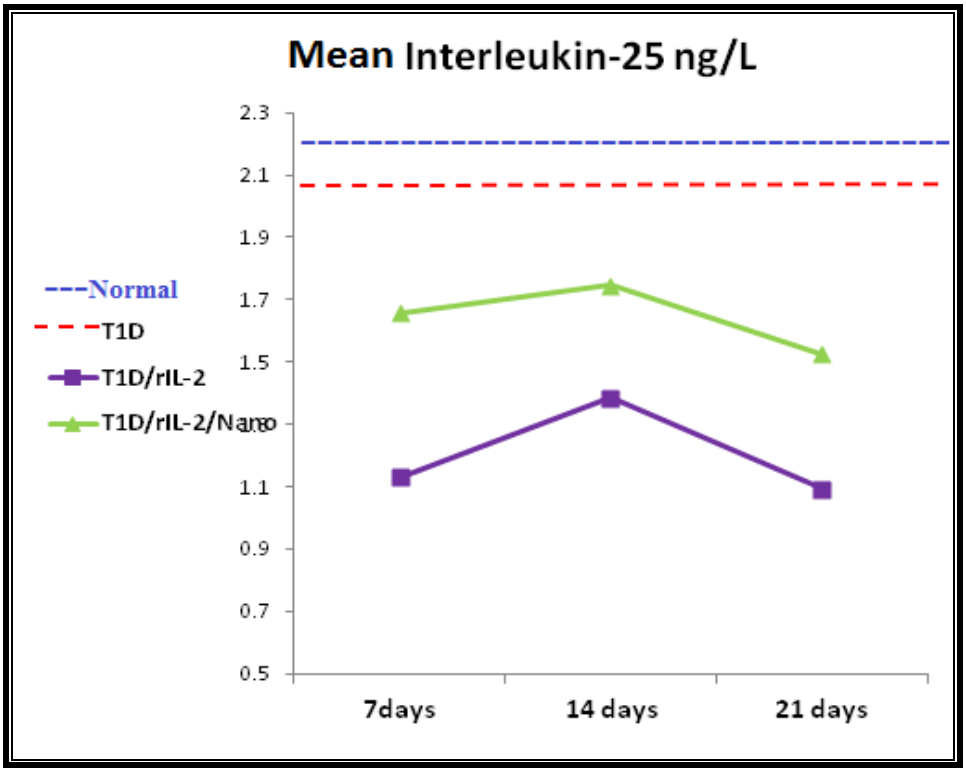

Figure (17): Time variation of IL-25 level through 21 days in T1D/rIL-2 and T1D/rIL-2/Nano groups.

DISCUSSION

Cytokines are likely to be involved in normal turnover and regeneration of β cells as well as in either immune homeostasis or alleviation of autoimmune reactions preceding T1D (Bonner-Weir et al., 2010). IL-23 is able to suppress Treg promoting the response of Th1 and Th17 and initiating chronic inflammation in several autoimmune diseases (Villanueva, 2017). IL-25, on the other hand, enhances Th2 immune polarization and participates in suppression of chronic autoimmune inflammation (Iwakura et al., 2011). This counter regulatory role of inflammatory cytokines in the pathogenesis and regulation of T1D pose considerable challenges for relevant therapeutic immune modulation. We intended to evaluate the modulatory effects of chitosan nanosphere encapsulated versus the free form of ultra low-doses of rhIL-2 during inflammatory consequences of experimental T1D.

Diabetic status was assessed through fasting blood glucose and serum insulin. Immunological abnormalities were monitored by measuring IL-23 and IL-25 in culture supernatants of short term splenocytes cultures. Our data revealed that treatment of diabetic mice with ultra low dose of rhIL-2, either free or encapsulated, improved the diabetic status

(manifested by decrease in blood glucose and increase in serum insulin) by the end of the first week after treatment. Unfortunately, this protective anti-diabetogenic effect of extrinsic low dose IL-2 did not show up at week 2. Interestingly, the therapeutic effect of chitosan-loaded, but not free IL-2, became much more pronounced by the end of the 3 weeks study; however, significant correlation with serum insulin level was collectively missing.

Since the first observation by Rossini et al., 1977 that MLD-STZ induce a sustained, yet transient, hyperglycaemia, hypo-insulinaemia and islet cell antibody positivity, experimental diabetic models have been extensively employed in research studies. The decreased hypoglycemic effect observed in the current study as compared to **T1D** group in diabetic mice 2 weeks after treatment with both forms of rhIL-2 may be attributed to the inability of ultra low dose rhIL-2 immunotherapy to improve glucose metabolic pathways such as glucose tolerance and liver glucose metabolism (Baeyens et al., 2013). This observation can also be attributed to an interesting finding of Pérol et al., (2016) who proved that rhIL-2 treatment may develop specific antibodies which are able to neutralize the injected rhIL-2 in mice. It seems likely that, prolonged retention of rhIL-2 by chitosan nanoparticles may perpetuate anti-rhIL-2 antibodies explaining the less pronounced effect of loaded rhIL-2 on reversal of T1D than the free form at this time. By day 21 post-treatment, the hypoglycemic effect of loaded rhIL-2 is rendered more pronounced than free rhIL-2, although this was not accompanied by decreased insulin level. It appears that the potential role of CS in alleviating T1D hyperglycemia through the decrease in liver gluconeogenesis and increase in skeletal muscle glucose uptake and use (Liu et al., 2015; Liu et al., 2010) became prominent at this phase. In addition, Liu et al., (2015) proved that dietary supplementation with CS significantly increased muscle glucose uptake-related signals including protein kinase B phosphorylation and GLUT4 translocation from the cytosol to cell membrane in the skeletal muscles of diabetic rats. These results could reflect the amelioration of diabetic state as well as increased insulin sensitivity and insulin effectiveness by supplementation with CS.

The potential involvement of IL-2/IL-2R in pathogenesis and/or regression of autoimmune inflammatory disorders represented an interesting area of research (Collison, 2019). It was previously evidenced that development of T1D is associated with either deficiency in IL-2 (Diaz-de-Durana et al., 2013) or in the allelic form of IL-2 receptor alpha (associated with Treg dysfunction) (Alcina et al., 2009; Vella et al., 2005). Recently, Abbas

et al., (2018) attributed the selective sensitivity of Treg cells to low dose IL-2 to constitutive expression of high affinity IL-2R.

In addition, NOD mice have relative IL-2 deficiency due to a genetic defect mapped to insulin-dependent diabetes (*Idd*)3 locus on chromosome 3 (Denny et al., 1997; Yamanouchi et al., 2007). Therapeutic administration of low-dose IL-2 has gained attention due to its capacity to boost Treg without the unwanted stimulation of Teff (Dwyer et al., 2016). This approach was effective not only in preventing T1D (Tang et al., 2008) but also to treat recent-onset T1D in NOD mice (Grinberg-Bleyer et al., 2010). A 5-day administration of low-dose IL-2 stimulated pancreatic frequency of Tregs and down regulated IFN-γ production by islet-infiltrating Teff inducing long-lasting diabetes remission. Tang et al., (2008), found that only 5 days of low-dose IL-2 administration to young pre-diabetic NOD mice prevents diabetes development remarkably. Grinberg-Bleyer et al., 2010 revealed that short-term administration of low-dose IL-2 into NOD mice induced long-lasting diabetes remission although inefficient in Treg-deficient mice indicating that Treg populations should be specifically stimulated in the pancreas in order to dampen the inflammatory milieu. Jailwala et al., 2009 proved that IL-2 deprivation induced apoptosis in Treg cells of T1D patients and that IL-2 infusion, signaling via STAT5, induced the expression of the anti-apoptotic molecules Bcl2 and Mcl1, preventing Treg apoptosis. Although transcriptome analyses by Grinberg-Bleyer et al., (2010) confirmed that extrinsic low-dose IL-2 almost exclusively acts on Treg, remission was established in only 60% of treated mice, and even half of them relapsed during the following months (Grinberg-Bleyer et al., 2010).

Our study showed that both free and chitosan-loaded rhIL-2 significantly impaired the splenocyte-derived IL-23 particularly at day 21. In addition, both rhIL-2 regimens induced a similar reduction in IL-25 expression as early as 7 and 14 days. However, this effect was prolonged till the end of the study only by free rather than chitosan-loaded IL-2 where the later had almost no effect.

It was recorded by Tang et al., (2012) that experimental T1D induction using sub-diabetogenic doses of MLD-STZ was not successful unless *in vivo* administration of IL-23. Extrinsic IL-23 was associated also with expression of IFN-γ and IL-17 in pancreatic islets and an augmentation of TNF-α and IL-18 expression increasing pancreatic cellular infiltration and β cell loss (Mensah-Brown et al., 2006). Moreover, suppression of IL-23 (or

its cognate receptor) or the IL-23 /IL-17 axis can be potential therapeutic targets in autoimmune diseases (Tang et al., 2012).

It has been postulated by Arce-Sillas et al., (2016) that activated DCs and macrophages are the main sources of IL-23 whereby inhibition of DCs by Tregs induced DCs inhibitory signals including down regulation of IL-23. This assumption was later enforced by Bauché et al., (2018) who proved that Treg inhibited IL-23 and IL-1β production from intestinal resident macrophages. In fact, DCs developed in the presence of IL-2 are more prone to apoptosis remaining either short-lived or attenuated with either pan or selective impairment of inflammatory cytokine production including IL-23 (Lau-Kilby et al., 2011). Moreover, *in vitro* stimulation of splenocytes following IL-2 treatment augment the secretion of regulatory cytokines such as IL-10, IL-4 and IL-5 leading to reduced expression of IL-23 in culture supernatants (Guerrero et al., 2014). The authors suggested that IL-2 inhibit the expression of regulatory molecules required for DC development, differentiation and STAT3 phosphorylation as well as increase the expression of suppressor of cytokine signaling (Socs) 1 and 3 known to be among potent negative regulators of DC function.

As an explanation for impaired IL-25 secretion following rhIL-2, it has been observed by Nelson et al., (1994) that low dose IL-2 treatment can induce alternatively activated macrophages (M2) to produce TGF-β that targets Th2 cells inhibiting IL-25 by a Sox4 and GATA3 dependent mechanism (Kuwahara et al., 2012) (Shapouri-Moghaddam et al., 2018). Moreover, Doherty et al., 2017 explained that Treg populations induced by low dose IL-2 can inhibit innate lymphoid cells (ILC-2) partially through production of IL-10 and TGF-β thereby, inhibiting IL-25 production (Doherty et al., 2017).

Interestingly, slightly higher doses of IL-2 resulted in a shift from immune tolerance to overt destructive autoimmunity (Baeyens et al., 2013). Subsequently, optimizing the current IL-2 therapeutic approaches (in terms of dosing and/or altered combinations that ensure slow sustained delivery of IL-2 to the proper target) could be of great importance re-orienting this approach towards the human disease (Pearson et al., 2017). In addition, the direct effect of low-dose IL-2 on the inflammatory process in T1D has not yet been fully elucidated. The effect of low-dose IL-2 therapy on the interplay of pro- and anti-inflammatory cytokines could help in proper interpretation (and subsequently, manipulation) of the autoimmune inflammatory disorders particularly T1D.

References

- Abbas, A. K., Trotta, E., Simeonov, D. R., Marson, A., & Bluestone, J. A. (2018). Revisiting IL-2: Biology and therapeutic prospects. *Science Immunology*, *3*(25). https://doi.org/10.1126/sciimmunol.aat1482
- Alcina, A., Fedetz, M., Ndagire, D., Fernández, O., Leyva, L., Guerrero, M., … Matesanz, F. (2009). IL2RA/CD25 gene polymorphisms: Uneven association with multiple sclerosis (MS) and type 1 diabetes (T1D). *PLoS ONE*, *4*(1), 1–5.
- Arce-Sillas, A., Álvarez-Luquín, D. D., Tamaya-Domínguez, B., Gomez-Fuentes, S., Trejo-Garciá, A., Melo-Salas, M., … Adalid-Peralta, L. (2016). Regulatory T Cells: Molecular Actions on Effector Cells in Immune Regulation. *Journal of Immunology Research*, *2016*. https://doi.org/10.1155/2016/1720827
- Baeyens, A., Pérol, L., Fourcade, G., Cagnard, N., Carpentier, W., Woytschak, J., … Piaggio, E. (2013). Limitations of IL-2 and rapamycin in immunotherapy of type 1 diabetes. *Diabetes*, *62*(9), 3120–3131.
- Bauché, D., Joyce-Shaikh, B., Jain, R., Grein, J., Ku, K. S., Blumenschein, W. M., … Cua, D. J. (2018). LAG3 + Regulatory T Cells Restrain Interleukin-23-Producing CX3CR1 + Gut-Resident Macrophages during Group 3 Innate Lymphoid Cell-Driven Colitis. *Immunity*, *49*(2), 342-352.e5.
- Bonner-Weir, S., Li, W. C., Ouziel-Yahalom, L., Guo, L., Weir, G. C., & Sharma, A. (2010). β-cell growth and regeneration: Replication is only part of the story. *Diabetes*, *59*(10), 2340–2348.
- Clark, M., Kroger, C. J., & Tisch, R. M. (2017). Type 1 diabetes: A chronic anti-self-inflammatory response. *Frontiers in Immunology*, *8*(DEC). https://doi.org/10.3389/fimmu.2017.01898
- Collison, J. (2019). Low-dose IL-2 therapy for autoimmune diseases. *Nature Reviews Rheumatology*, *15*(1), 2. https://doi.org/10.1038/s41584-018-0142-1
- Denny, P., Lord, C. J., Hill, N. J., Goy, J. V., Levy, E. R., Podolin, P. L., … Lyons, P. A. (1997). Mapping of the IDDM locus Idd3 to a 0.35-cM interval containing the interleukin-2 gene. *Diabetes*, *46*(4), 695–700.
-

© GSJ

–Diaz-de-Durana, Y., Lau, J., Knee, D., Filippi, C., Londei, M., McNamara, P., … Herman, A. E. (2013). IL-2 Immunotherapy Reveals Potential for Innate Beta Cell Regeneration in the Non-Obese Diabetic Mouse Model of Autoimmune Diabetes. *PLoS ONE*, *8*(10), 1–12.

–Doherty, T. A., & Broide, D. H. (2017). Pathways to limit group 2 innate lymphoid cell activation. *Journal of Allergy and Clinical Immunology*, *139*(5), 1465–1467.

–Duvallet, E., Semerano, L., Assier, E., Falgarone, G., & Boissier, M. C. (2011). Interleukin-23: A key cytokine in inflammatory diseases. *Annals of Medicine*, *43*(7), 503–511.

–Dwyer, C. J., Ward, N. C., Pugliese, A., & Malek, T. R. (2016). Promoting Immune Regulation in Type 1 Diabetes Using Low-Dose Interleukin-2. *Current Diabetes Reports*, *16*(6). https://doi.org/10.1007/s11892-016-0739-1

–Fort, M. M., Cheung, J., Yen, D., Li, J., Zurawski, S. M., Lo, S., … Rennick, D. M. (2001). IL-25 Induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity*, *15*(6), 985–995.

–Furman, B. L. (2015). Streptozotocin-Induced Diabetic Models in Mice and Rats Streptozotocin-Induced Diabetic Models. *Curr. Protoc. Pharmacol*, *70*, 1–20.

–Grinberg-Bleyer, Y., Baeyens, A., You, S., Elhage, R., Fourcade, G., Gregoire, S.,… Piaggio, E. (2010). IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *Journal of Experimental Medicine*, *207*(9), 1871-1878.

–Guerrero, A. D., Dong, M. B., Zhao, Y., Lau-Kilby, A., & Tarbell, K. V. (2014). Interleukin-2-mediated inhibition of dendritic cell development correlates with decreased CD135 expression and increased monocyte/macrophage precursors. *Immunology*, *143*(4), 640–650.

–Iwakura, Y., Ishigame, H., Saijo, S., & Nakae, S. (2011). Functional Specialization of Interleukin-17 Family Members. *Immunity*, *34*(2), 149–162.

–Jailwala, P., Waukau, J., Glisic, S., Jana, S., Ehlenbach, S., Hessner, M., … Ghosh, S. (2009). Apoptosis of CD4+CD25high T cells in type 1 diabetes may be partially mediated by IL-2 deprivation. *PLoS ONE*, *4*(8). https://doi.org/10.1371/journal.pone.0006527

–Kuwahara, M., Yamashita, M., Shinoda, K., Tofukuji, S., Onodera, A., Shinnakasu, R., … Nakayama, T. (2012). The transcription factor Sox4 is a downstream target of signaling by the cytokine TGF-β and suppresses Th2 differentiation. *Nature Immunology*, *13*(8), 778–

786.

–Lau-Kilby, A. W., Kretz, C. C., Pechhold, S., Price, J. D., Dorta, S., Ramos, H., … Tarbell, K. V. (2011). Interleukin-2 inhibits FMS-like tyrosine kinase 3 receptor ligand (flt3L)-dependent development and function of conventional and plasmacytoid dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(6), 2408–2413.

–Liu, G., Chen, S., Guan, G., Tan, J., Al-Dhabi, N. A., Wang, H., … Fang, J. (2016). Chitosan Modulates Inflammatory Responses in Rats Infected with Enterotoxigenic Escherichia coli. *Mediators of Inflammation*, *2016*. https://doi.org/10.1155/2016/7432845

–Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, C. F., & Randolph GJ, Rudensky AY, N. M. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science*, *324*(5925), 392–397.

–Liu, S. H., Cai, F. Y., & Chiang, M. T. (2015). Long-term feeding of chitosan ameliorates glucose and lipid metabolismin a high-fructose-diet-impaired rat model of glucose tolerance. *Marine Drugs*, *13*(12), 7302–7313.

–Liu, S. H., Chang, Y. H., & Chiang, M. T. (2010). Chitosan reduces gluconeogenesis and increases glucose uptake in skeletal muscle in streptozotocin-induced diabetic rats. *Journal of Agricultural and Food Chemistry*, *58*(9), 5795–5800.

–Mensah-Brown, E. P. K., Shahin, A., Al-Shamisi, M., Wei, X., & Lukic, M. L. (2006). IL-23 leads to diabetes induction after subdiabetogenic treatment with multiple low doses of streptozotocin. *European Journal of Immunology*, *36*(1), 216–223.

–Mohammadpour Dounighi N, Eskandari R, Avadi MR, Zolfagharian H, Mir Mohammad Sadeghi A, R. M. (2012). Preparation and in vitro characterization of chitosan nanoparticles containing Mesobuthus eupeus scorpion venom as an antigen delivery system. *J Venom Anim Toxins Incl Trop Dis*, *18*(1), 44–52.

–Mohammed, M. A., Syeda, J. T. M., Wasan, K. M., & Wasan, E. K. (2017). An overview of chitosan nanoparticles and its application in non-parenteral drug delivery. *Pharmaceutics*, *9*(4). https://doi.org/10.3390/pharmaceutics9040053

–Nelson BJ, Danielpour D, Rossio JL, Turpin J, N. C. (1994). Interleukin-2 suppresses activated macrophage intracellular killing activity by inducing macrophages to secrete TGF-beta. *Leukoc Biol*, 55(1):81- 90.

–Pearson, R. M., Casey, L. M., Hughes, K. R., Wang, L. Z., North, M. G., Getts, D. R., … Shea, L. D. (2017). Controlled Delivery of Single or Multiple Antigens in Tolerogenic Nanoparticles Using Peptide-Polymer Bioconjugates. *Molecular Therapy*, *25*(7), 1655–1664.

–Pérol, L., Lindner, J. M., Caudana, P., Nunez, N. G., Baeyens, A., Valle, A., … Piaggio, E. (2016). Loss of immune tolerance to IL-2 in type 1 diabetes. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms13027

–Rossini, A. A., Like, A. A., Chick, W. L., Appel, M. C., & Cahill, G. F. (1977). Studies of streptozotocin induced insulitis and diabetes. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(6), 2485–2489.

–Shapouri-Moghaddam, A., Mohammadian, S., Vazini, H., Taghadosi, M., Esmaeili, S. A., Mardani, F., … Sahebkar, A. (2018). Macrophage plasticity, polarization, and function in health and disease. *Journal of Cellular Physiology*, *233*(9), 6425–6440.

–Singh, B., Nikoopour, E., Huszarik, K., Elliott, J. F., & Jevnikar, A. M. (2011). Immunomodulation and regeneration of islet beta cells by cytokines in autoimmune type 1 diabetes. *Journal of Interferon and Cytokine Research*, *31*(10), 711–719.

–Stosic-Grujicic, S., Cvetkovic, I., Mangano, K., Fresta, M., Maksimovic-Ivanic, D., Harhaji, L., … Nicoletti, F. (2007). A potent immunomodulatory compound, (S,R)-3-Phenyl-4,5-dihydro-5-isoxasole acetic acid, prevents spontaneous and accelerated forms of autoimmune diabetes in NOD mice and inhibits the immunoinflammatory diabetes induced by multiple low doses of streptozoto. *Journal of Pharmacology and Experimental Therapeutics*, *320*(3), 1038–1049.

–Stošić-Grujičić, S. D., Maksimović, D. D., Stojković, M. B. M., & Lukic, M. L. (2001). Pentoxifylline prevents autoimmune mediated inflammation in low dose streptozotocin induced diabetes. *Developmental Immunology*, *8*(3–4), 213–221.

–Tang, C., Chen, S., Qian, H., & Huang, W. (2012). Interleukin-23: As a drug target for autoimmune inflammatory diseases. *Immunology*, *135*(2), 112–124.

–Tang, Q., Adams, J. Y., Penaranda, C., Melli, K., Piaggio, E., Sgouroudis, E., … Bluestone, J. A. (2008a). *Central Role of Defective Interleukin-2 Production in the Triggering of Islet Autoimmune Destruction*. (May), 687–697.

© GSJ

– Tang, Q., Adams, J. Y., Penaranda, C., Melli, K., Piaggio, E., Sgouroudis, E., … Bluestone, J. A. (2008b). Central Role of Defective Interleukin-2 Production in the Triggering of Islet Autoimmune Destruction. *Immunity*, *28*(5), 687–697.

– Vella, A., Cooper, J. D., Lowe, C. E., Walker, N., Nutland, S., Widmer, B., … Todd, J. A. (2005). Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms. *American Journal of Human Genetics*, *76*(5), 773–779.

– Villanueva, M. T. (2017). Rheumatoid arthritis: IL-23 assists the transition from autoimmunity to inflammatory disease. *Nature Reviews Rheumatology*, *13*(1), 1. https://doi.org/10.1038/nrrheum.2016.197

– Yamanouchi, J., Rainbow, D., Serra, P., Howlett, S., Hunter, K., Garner, V. E. S., … Santamaria, P. (2007). Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nature Genetics*, *39*(3), 329–337.

– Wu, J., & Yan, L. J. (2015). Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic β cell glucotoxicity. *Diabetes, metabolic syndrome and obesity: targets and therapy*, *8*, 181–188.

© GSJ