Original Article

Chimeric ScFv Monoclonal Antibody Composed of Tocilizumab-Light Chain, Heavy Chain, and Bacterial Flagellin for the Treatment of Autoimmune Disorder: Insilco Design and Cytotoxicity Study

K Rajaganapathy[*1], K Masilamani[2], B Senthilnathan[2], Vasanth Kumar M[3], Suthakaran P [4], Panneerselvam P [2] , Vigneshwar M[5], Prashantha CN[6].

[*1]Bioreach Labs (OPC) Pvt Ltd, No-163, 8th Cross Street, Balaji Nagar, Madampakkam, Chennai-600126
[2]Faculty of Pharmacy, Sree Balaji Medical College and Hospital Campus, Bharath Institute of Higher Education and Research, Chromepet, Chennai 600044
[3]Faculty of Pharmacy, Bharath Institute of Higher Education and Research, Selaiyur, East Tambaram, Chennai-600073
[4]Genprotic Biopharma Pvt Ltd, Research centre held at E-Yuva Centre, Anna University, Taramani Campus, Taramani, Chennai, Tamil Nadu 600113.
[5]Department of Pharmaceutics, Faculty of Pharmacy, Dr.MGR Educational and Research Institute,Velappanchavadi Chennai 600077.
[6]Department of Biotechnology, School of Applied Sciences, Reva University, Bangalore-560064.
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Abstract

Introduction: The Tocilizumab (TCZ), a recombinant humanised anti-interleukin-6 receptor (IL-6R) monoclonal antibody, is primarily used to treat various autoimmune disorders specifically, rheumatoid arthritis. The usage of tocilizumab injection may reduce our capacity to combat infections brought on by bacteria, viruses, and fungi and raise our risk of developing a serious or potentially fatal illness that may spread throughout the body. To overcome these obstacles, the current work was concentrate on the Designing of a Chimeric Flagellin-mediated Tocilizumab Monoclonal Antibody for Autoimmune Disorders. The flagellum, a whip-like appendage that facilitates bacterial mobility, has a component protein called flagellin which has now been discovered to be a potent immune activator that shapes both the innate and adaptive immune systems in response to microbial infections.

Methods: In this study, we were developed an In-silico, methods to design Chimeric-ScFv Monoclonal antibody which was based on speciallized linker to connect bacterial flagellin to Tocilizumab, between the heavy and light chains, yielding a chimeric antibody. Further the preliminary In-vitro cytotoxicity study attempt was on carried out on the MTT Viability assay of Chondrocytes and Fibroblasts in RA co-cultures with TCZ, Flagelline and TCZ+Flagelline Coadminstration.

Results: Our findings suggest that the Flagelline mediated Tocilizumab monoclonal antibody, which was created by in-silico methods using chimeric ScFv, has good solubility, stability, and agonist and antagonist effects on TLR-5 and IL-6 respectivelly, based on binding and activation. The preliminary cytotoxicity study indicating the most active inhibition effects on fibroblast and chondrocytes with Co-adminstration of TCZ+Flagelline.

Conclusion: Under this Proof of concept, the focus of future work will be based on the development, cloning, protein purification, characterization, and assessment of Chimeric flagelline mediated Tocilizumab monoclonal antibody for the treatment autoimmune disorders.

KeyWords: Autoimmune, Chimeric Protein, ScFv, Tocilizumab, Monoclonal Antibody, In-silico and Cytotoxicity.

Introduction:

A pathologic condition known as autoimmunity occurs when the body's own tissues are attacked by immunological responses, resulting in tissue destruction (autoimmunity). There are currently around 80 autoimmune disorders understood. While some are common and easy to identify, like type 1 diabetes, multiple sclerosis, lupus, and rheumatoid arthritis, others are uncommon and challenging. A global estimate indicates that about 700 million people, or almost 10 percent of the world's population, have autoimmune diseases of varying severity (1). Tocilizumab (TCZ), a recombinant humanised anti-interleukin-6 receptor (IL-6R)

monoclonal antibody, is primarily used to treat various autoimmune disorders specifically, rheumatoid arthritis, systemic juvenile idiopathic arthritis (sJIA), and polyarticular juvenile idiopathic arthritis (pJIA). The usage of tocilizumab injection may reduce our capacity to combat infections brought on by bacteria, viruses, and fungi and raise our risk of developing a serious or potentially fatal illness that may spread throughout the body (1). To overcome these obstacles, the current work was concentrate on the Designing of a Chimeric Flagellin-mediated Tocilizumab Monoclonal Antibody for Autoimmune Disorders. The flagellum, a whip-like appendage that facilitates bacterial mobility, has a component protein called flagellin which has now been discovered to be a potent immune activator that shapes both the innate and adaptive immune systems in response to microbial infections.

In this study, we were developed an In-silico, methods to design Chimeric-ScFv Monoclonal antibody which was based on speciallized linker to connect bacterial flagellin to Tocilizumab, between the heavy and light chains, yielding a chimeric antibody (2-6).

The chimeric-ScFv constructs comprised of Tocilizumab light chain connected with Tocilizumab Heavy chain via a flexible linker like **GGGGSGGGGSGGGGS**, as a targeting molecule of Single chain variable fragments (2-6). The length and amino acid composition of the peptide linker is important in maintaining the scFv structure and stability. Typically, due to its flexibility, the linker peptide is about 3.5 nm (35°A) in length and contains hydrophobic amino acids such as glycine and serine residues. Furthered the bacterial flagellar antigens FliC (from Escherichia coli) which were conjugated via specific flexible linker **RGRR** (It's a linker derived from furin protease recognition site) finally obtained the Chimeric ScFv (2-6). A 3D-homology model for this chimeric protein were constructed and its structure, stability, solubility and binding to IL-6 and TLR-5 were predicted with the help of Insilico methods then, evaluated, using, In-vitro cytotoxicity studies were promote the proliferation and growth of chondrocytes and fibroblasts in rheumatoid arthritis Co-cultures by using various concentration of TCZ, Flagellin and TCZ + Flagelline Co-admistration for the purposes of constructing **Chimeric Flagellin-mediated Tocilizumab Monoclonal Antibody for Autoimmune Disorders**.

Materials and Methods

The Tocilizumap and Flagellin from E.Coli were purchased from Sigma-Aldrichand the in-silico chimeric-ScFv constructs (Shown Table-1and Figure-1) comprised of Tocilizumab light chain connected with Tocilizumab Heavy chain (Retrieved from pubchem) via a flexible linker like GGGGSGGGGSGGGGS, as a targeting molecule of Single chain variable fragments. The length and amino acid composition of the peptide linker is important in maintaining the scFv structure and stability. Typically, due to its flexibility, the linker peptide is about 3.5 nm (35°A) in length and contains hydrophobic amino acids such as glycine and serine residues. Furthered the bacterial flagellar antigens FliC (from Escherichia coli) were retrieved from NCBI (Genbank ID: AAW84044.1) which were conjugated via specific flexible linker RGRR (It's a linker derived from furin protease recognition site) finally we obtain a Chimeric ScFv (2-6). A 3D-homology model for this chimeric protein were constructed and its solubility, stability and binding to IL-6 and TLR-5 were predicted with the aim of the pharmacological activity for auto immune disorders and then, evaluated, using In-silico and In-vitro procedures.

Protein-Peptide Docking:

CABS: A set of tools for protein-peptide docking was a CABS programming of the fast protocol which uses flexible docking for protein-peptide interactions (6). The chimeric antibody like Tocilizumab Flagelline were separately docked with IL-6, TLR-5 and TLR-3 and its solubility and stability also were evaluated.

In-vitro Cytotoxicity with Chondrocytes and Fibroblasts in rheumatoid arthritis Co-cultures:

MTT and AO/EB assays were used to determine the cytotoxicity of samples such as tocilizumab (TCZ), flagelline (FLAGLN), and TCZ + FLAGLN. In a assays, chondrocytes and fibroblasts from rats were prepared using enzymatic digestion in accordance with the noted procedures (7-12). The cells were then seeded into a

petri dish and grown with Dulbecco's modified Eagle medium (DMEM)/F-12 medium with 10% (v/v) fetal bovine serum at 37 °C and 5% CO2. Every two days, the medium was changed. After being digested, the cultivated cells were diluted to 5 × 104 cells/mL. The produced solutions were applied to a 96-well plate with 100 µL per well and then co-cultured with cells for 24 hours at sample concentrations of 10%, 25%, 50%, and 100%. The prepared and irradiated (UV, 12 h) samples were then added one at a time, totaling 20 µL. 20 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution were introduced into each well at the indicated time points (1, 4 and 7 days), and the cells were subsequently grown for an additional 4 hours. After the culture was finished, the upper solution was discarded. After that, 150 µL of DMSO were added in order to dissolve the blue formazan reaction result. Three separate measurements of the solution's absorbance value were taken and recorded using a microplate reader (Bio-Rad iMark). Three parallel experiments were used to calculate the final results.

An AO/EB staining experiment was run to examine the samples' cytotoxicity in more detail. In a 24-well plate with a coverslip in each well, chondrocytes (3 × 104 cells/well) were planted. 10 µL of samples such as control, ASA, Flagelline, TCZ+ASA, TCZ+Flagelline, and TCZ were introduced separately and further cultured for various days after the cells were cultured for 24 hours and proliferated by static adhesion (1, 4, and 7 days). Coverslips containing cells were rinsed three times with PBS (pH=7.4), followed by the addition of 20 L of the AO/EB solution and gentle mixing at the designated time points. The coverslips were placed on microscope slides after 5 minutes and rinsed with PBS (pH = 7.4). A fluorescent microscope was used to take the pictures, and Image J was used to analyse them (7-12).

Results:

Table 1: The amino acid sequence of construct of Chimeric Flagellin-mediated Tocilizumab Monoclonal Antibody

>Tocilizumab light chain:
DIQMTQSPSSLSASVGDRVTITCRASQDISSYLNWYQQKPGKAPKLLIYYTSRLHSGVPSRFSGSGSGTD
FTFTISSLQPEDIATYYCQQGNTLPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP
REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF
NRGEC**GGGGSGGGGSGGGGS**>**Tocilizumab heavy chain:**
QVQLQESGPGLVRPSQTLSLTCTVSGYSITSDHAWSWVRQPPGRGLEWIGYISYSGITTYNPSLKSRVT
MLRDTSKNQFSLRLSSVTAADTAVYYCARSLARTTAMDYWGQGSLVTVSSASTKGPSVFPLAPSSKST
SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG**RGRR**>sp|P04949|FLIC_ECOLI Flagellin OS=Escherichia coli (strain K12) OX=83333 GN=fliC PE=1 SV=2MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANRFTSNIKGLTQAARN
ANDGISVAQTTEGALSEINNNLQRVRELTVQATTGTNSESDLSSIQDEIKSRLDEIDRVSGQTQFNGVNV
LAKNGSMKIQVGANDNQTITIDLKQIDAKTLGLDGFSVKNNDTVTTSAPVTAFGATTTNNIKLTGITLST
EAATDTGGTNPASIEGVYTDNGNDYYAKITGGDNDGKYYAVTVANDGTVTMATGATANATVTDANT
TKATTITSGGTPVQIDNTAGSATANLGAVSLVKLQDSKGNDTDTYALKDTNGNLYAADVNETTGAVS
VKTITYTDSSGAASSPTAVKLGGDDGKTEVVDIDGKTYDSADLNGGNLQTGLTAGGEALTAVANGKT
TDPLKALDDAIASVDKFRSSLGAVQNRLDSAVTNLNNTTTNLSEAQSRIQDADYATEVSNMSKAQIIQQ
AGNSVLAKANQVPQQVLSLLQG

V_L	**Flexible Linker**	**V_H**	**Linker It has been derived from Furin proteases recognition site**	**flagellin (FliC)-from Escherichia coli**

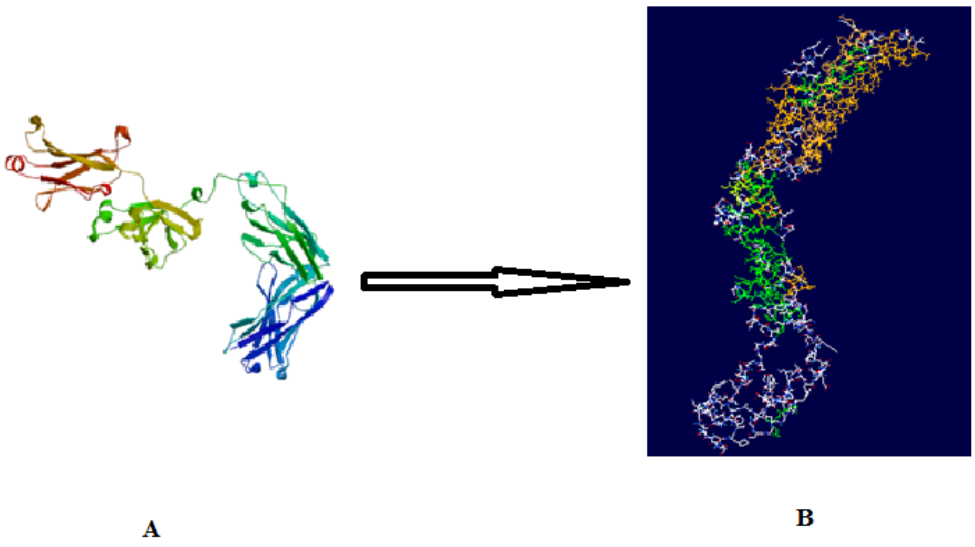

Figure-1: 3D models of mimicked chimeric-ScFv constructs comprised of Flagellin from E.Coli mediated Tocilizumab monoclonal antibody: A). Secondary structure of antibody- Antigen complex; B) Refined model, Green colour shown the Tocilizumab V-Light Chain, Reddish yellow shown the Tocilizumab V-Heavy chain and White shown the flagellin (FliC)-from Escherichia coli.

The ten 3D models of mimicked chimeric-ScFv constructs (Shown Table-1and Figure-1) comprised of Flagellin from E.Coli mediated Tocilizumab monoclonal antibody were generated by the CABS dock server. The Antibody-Antigen Complex chimeric protein was bound to the both IL-6, TLR-3 and TLR-5 receptor with high antagonistic affinity and specificity and also consequently the specific Antibody-Antigen Complex chimeric protein were bound with immunostimulant activity of TLR-5. One of the best-visualized models for this docking was illustrated in **Figure-2-5 and Table-2-9** along with best interactions contains various interfaces region, ligand binding amino acids and docking score also were illustrated.

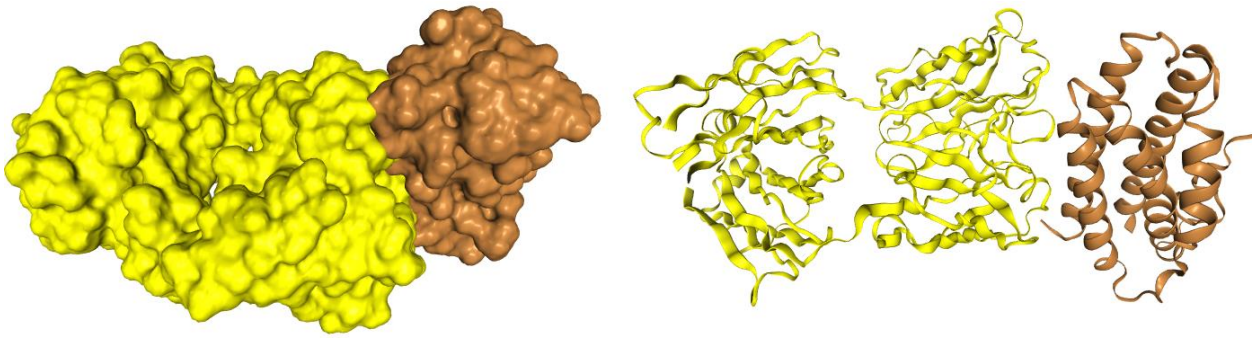

Figure-2: TLR-5 with Chimeric ScFv McAB.

Table-2 Docking Score of TLR-**5 with Chimeric ScFv McAB.**

Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Docking Score	-256.50	-241.58	-226.95	-222.04	-219.35	-218.13	-217.63	-216.48	-211.64	-211.41
Ligand RMSD	73.25	54.09	75.82	53.64	73.11	39.55	58.29	45.43	41.91	75.07

(Å)										

Table-3 Amino Acids Interaction of TLR-5 with Chimeric ScFv McAB.

Receptor interface residue(s):	Ligand interface residue(s):	Receptor-ligand interface residue pair(s):
LEU 19A 2.394	SER 31H 3.664	19A - 2L 4.729
ARG 24A 2.740	TRP 101H 3.106	19A - 27L 2.394
LYS 27A 2.171	LEU 102H 1.941	19A - 28L 3.466
GLN 28A 2.031	GLY 103H 4.169	24A - 28L 2.740
ILE 29A 4.446	PRO 104H 4.513	24A - 29L 4.192
TYR 31A 1.941	ILE 2L 4.729	24A - 30L 3.815
ILE 32A 4.921	GLN 27L 2.394	24A - 33L 4.897
ASP 34A 4.668	SER 28L 2.740	24A - 69L 3.755
GLY 35A 3.488	VAL 29L 4.192	27A - 33L 2.171
ALA 38A 3.511	GLY 30L 3.455	27A - 93L 3.231
LYS 41A 3.106	SER 31L 3.199	27A - 94L 3.024
GLU 110A 2.053	SER 32L 2.031	28A - 30L 3.982
GLN 111A 3.482	TYR 33L 2.171	28A - 31L 4.282
ARG 113A 2.611	TYR 50L 3.009	28A - 32L 2.031
ALA 114A 2.996	GLY 51L 4.781	28A - 33L 2.571
VAL 115A 4.902	PHE 53L 3.396	29A - 33L 4.446
MET 117A 3.741	SER 54L 2.611	31A - 102H 1.941
SER 118A 2.656	ARG 55L 2.861	31A - 103H 4.169
VAL 121A 3.199	ALA 56L 3.148	31A - 104H 4.513
GLN 124A 4.679	THR 57L 2.053	31A - 32L 2.790
PHE 125A 3.455	GLY 58L 4.805	31A - 33L 2.567
LYS 128A 4.803	SER 68L 4.679	31A - 50L 4.551
	GLY 69L 3.755	31A - 51L 4.781
	TYR 92L 3.140	31A - 92L 3.140
	GLY 93L 3.231	32A - 32L 4.921
	SER 94L 3.024	32A - 33L 4.982
		34A - 101H 4.668
		35A - 101H 4.952
		35A - 102H 3.488
		38A - 101H 3.511
		41A - 31H 3.664
		41A - 101H 3.106
		110A - 102H 4.456
		110A - 50L 3.009
		110A - 55L 2.861
		110A - 56L 3.148
		110A - 57L 2.053
		110A - 58L 4.805
		111A - 101H 3.482
		111A - 102H 3.642
		111A - 50L 4.239
		113A - 53L 3.619
		113A - 54L 2.611
		113A - 55L 4.083
		114A - 102H 2.996
		114A - 50L 3.613

		114A - 54L 4.245 115A - 102H 4.902 117A - 53L 3.741 117A - 54L 4.000 118A - 32L 2.656 121A - 30L 4.840 121A - 31L 3.199 121A - 32L 4.443 121A - 53L 3.396 124A - 68L 4.679 125A - 29L 4.590 125A - 30L 3.455 125A - 31L 3.806 128A - 68L 4.803

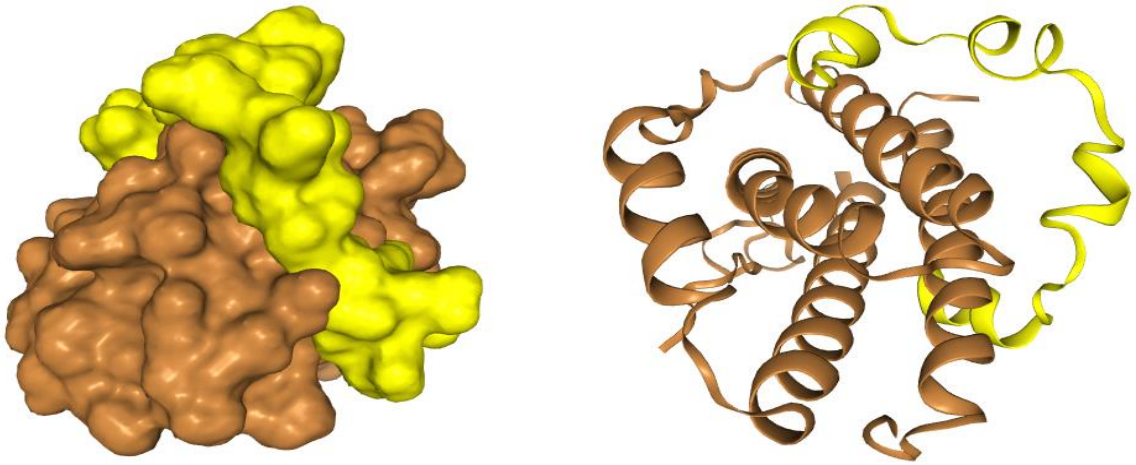

Figure-3: IL-6 with Flagellin

Table-4: Docking Score of IL-6 with Flagellin

Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Docking Score	-216.78	-211.90	-201.78	-195.42	-192.26	-189.21	-187.99	-187.30	-186.69	-186.15
Ligand RMSD (Å)	48.85	67.75	52.57	59.17	61.56	47.27	57.69	65.89	52.26	72.61

Table-5: Amino acids interactions of IL-6 with Flagellin

Receptor interface residue(s)	Ligand interface residue(s)	Receptor-ligand interface residue pair(s):
ILE 29A 4.837	VAL 476E 4.475	29A - 513E 4.837
ARG 30A 3.304	PHE 478E 2.493	30A - 513E 3.304
TYR 31A 2.920	ALA 479E 3.802	31A - 497E 2.920
LEU 33A 2.858	GLU 481E 2.704	33A - 512E 2.858
ASP 34A 2.409	SER 482E 1.199	33A - 513E 3.596
GLY 35A 4.104	ALA 483E 3.976	34A - 504E 4.345
ILE 36A 4.361	ASN 484E 4.740	34A - 509E 2.409
SER 37A 2.504	PHE 485E 2.968	34A - 512E 4.596
ALA 38A 3.768	ASN 489E 4.017	34A - 513E 4.390
ARG 40A 3.108	ILE 490E 4.179	35A - 501E 4.104
LYS 41A 2.993	GLN 493E 3.819	36A - 512E 4.361

GLU 95A 4.852	SER 494E 4.944	37A - 504E 4.923
GLN 102A 3.377	GLY 495E 4.107	37A - 505E 4.604
GLU 110A 4.107	PHE 497E 2.920	37A - 508E 2.773
GLN 111A 1.591	ALA 500E 3.009	37A - 509E 4.084
ARG 113A 2.704	GLN 501E 1.591	37A - 512E 2.504
ALA 114A 3.983	ALA 502E 4.911	38A - 501E 3.768
GLN 116A 3.357	ASN 503E 2.993	38A - 503E 4.594
MET 117A 2.767	ALA 504E 4.345	38A - 504E 4.719
SER 118A 4.889	VAL 505E 3.203	40A - 508E 3.108
LYS 120A 1.199	ASN 508E 2.773	41A - 503E 2.993
VAL 121A 2.968	VAL 509E 2.409	41A - 504E 4.867
GLN 124A 2.538	ARG 511E 2.513	41A - 505E 3.203
LYS 171A 2.581	LEU 512E 2.504	95A - 482E 4.852
GLN 175A 2.513	LEU 513E 3.304	102A - 478E 3.377
LEU 178A 4.060	GLN 514E 3.090	110A - 493E 4.957
ARG 179A 3.090		110A - 494E 4.944
		110A - 495E 4.107
		110A - 500E 4.117
		111A - 500E 3.009
		111A - 501E 1.591
		111A - 502E 4.911
		111A - 503E 4.828
		113A - 476E 4.475
		113A - 478E 2.937
		113A - 481E 2.704
		113A - 489E 4.017
		113A - 490E 4.689
		113A - 493E 3.819
		114A - 490E 4.179
		114A - 497E 4.420
		114A - 501E 3.983
		116A - 478E 3.357
		116A - 482E 4.856
		117A - 478E 2.938
		117A - 481E 2.767
		117A - 482E 4.539
		117A - 485E 3.008
		117A - 489E 4.914
		118A - 485E 4.889
		120A - 478E 2.493
		120A - 479E 3.802
		120A - 481E 4.971
		120A - 482E 1.199
		121A - 485E 2.968
		124A - 482E 2.538
		124A - 483E 3.976
		124A - 484E 4.740
		124A - 485E 4.216
		171A - 512E 2.581
		175A - 511E 2.513
		175A - 512E 2.668
		175A - 513E 3.941

		175A - 514E 4.393 178A - 513E 4.060 179A - 514E 3.090

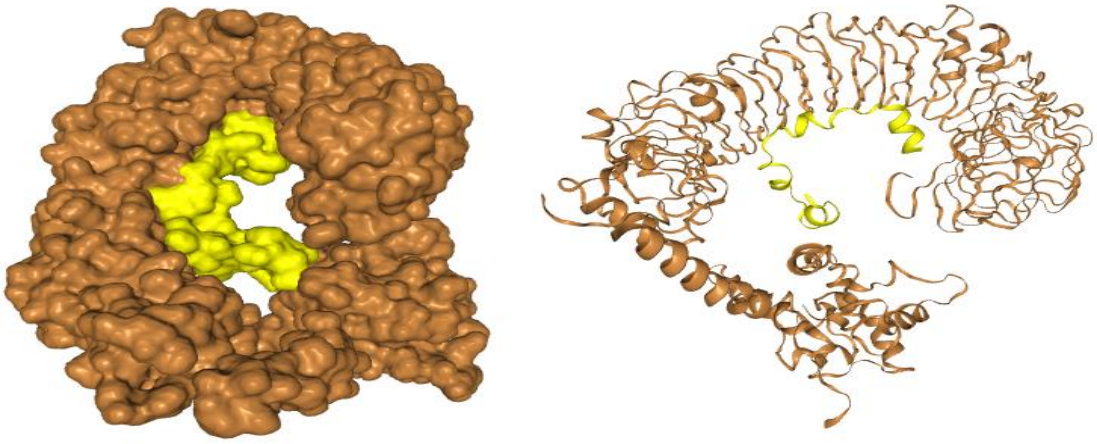

Figure-4: TLR-5 with Flagillin

Table-6: Docking Score of TLR-5 with Flagillin

Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Docking Score	-269.76	-258.60	-257.16	-251.96	-251.80	-247.38	-240.78	-237.55	-237.03	-236.73
Ligand rmsd (Å)	176.17	127.45	188.03	187.45	167.22	189.01	166.70	160.40	139.00	183.70

Table-7: Amino acid interactions of TLR-5 with Flagillin

Receptor interface residue(s):	Ligand interface residue(s):	Receptor-ligand interface residue pair(s):
LYS 173A 2.779	ASP 477E 4.184	173A - 514E 2.779
SER 174A 1.654	GLU 480E 3.496	174A - 511E 4.380
PHE 200A 3.010	ALA 483E 2.737	174A - 514E 1.654
PHE 201A 4.610	ASN 484E 2.632	200A - 514E 3.010
SER 202A 2.695	PHE 485E 4.900	201A - 514E 4.610
GLU 229A 3.249	LYS 487E 3.483	202A - 511E 4.812
ILE 230A 3.635	TYR 488E 4.249	202A - 514E 2.695
ASP 232A 2.944	LEU 491E 2.944	229A - 510E 4.157
PHE 256A 2.899	ALA 492E 4.130	229A - 514E 3.249
SER 257A 3.893	GLN 493E 3.445	230A - 507E 4.734
ILE 259A 3.509	SER 494E 3.107	230A - 510E 4.546
HIS 292A 2.451	GLY 495E 3.684	230A - 511E 3.635
ASP 294A 3.651	SER 496E 3.637	230A - 514E 4.430
SER 296A 4.847	ALA 498E 1.724	232A - 507E 3.061
VAL 316A 4.250	MET 499E 0.991	232A - 511E 2.944
LEU 317A 2.886	ALA 500E 4.209	256A - 510E 2.899
ASN 318A 2.402	GLN 501E 4.675	257A - 510E 3.893
ALA 320A 4.168	ALA 502E 3.067	259A - 507E 3.509
VAL 340A 2.799	ASN 503E 2.442	292A - 506E 2.451
LEU 341A 4.465	ALA 504E 4.378	292A - 507E 4.939
ASN 342A 2.483	VAL 505E 4.168	292A - 510E 3.503
ASP 366A 4.378	GLN 506E 2.402	294A - 506E 4.966

GLN 368A 2.442	GLN 507E 3.061	294A - 507E 3.651
ASP 390A 4.378	LEU 510E 2.899	296A - 507E 4.847
ARG 392A 2.466	ARG 511E 2.944	316A - 506E 4.250
PHE 409A 1.724	GLN 514E 1.654	317A - 506E 2.886
LEU 410A 4.292		318A - 505E 4.861
SER 411A 0.991		318A - 506E 2.402
GLY 412A 4.167		318A - 507E 3.988
HIS 429A 3.409		320A - 505E 4.168
LEU 430A 4.931		340A - 506E 2.799
SER 431A 3.520		341A - 506E 4.465
GLN 451A 3.483		342A - 505E 4.718
ILE 452A 3.204		342A - 506E 2.483
ILE 454A 3.684		366A - 504E 4.378
ASN 456A 3.107		368A - 502E 3.469
GLU 476A 4.010		368A - 503E 2.442
GLN 477A 2.944		390A - 502E 4.378
PHE 479A 3.091		392A - 498E 2.534
ARG 529A 4.670		392A - 499E 2.537
ASN 709A 4.758		392A - 500E 4.209
LYS 713A 2.632		392A - 501E 4.675
ASP 716A 4.886		392A - 502E 3.067
SER 720A 4.178		392A - 503E 2.466
ASP 721A 3.496		409A - 498E 1.724
		409A - 499E 4.431
		410A - 499E 4.292
		411A - 499E 0.991
		412A - 499E 4.167
		429A - 496E 4.428
		429A - 498E 3.409
		430A - 499E 4.931
		431A - 496E 3.637
		431A - 499E 3.520
		451A - 487E 3.483
		451A - 491E 4.853
		452A - 491E 3.204
		454A - 494E 4.610
		454A - 495E 3.684
		454A - 496E 4.171
		456A - 494E 3.107
		456A - 495E 3.974
		456A - 496E 4.662
		476A - 487E 4.010
		476A - 488E 4.249
		477A - 488E 4.880
		477A - 491E 2.944
		477A - 492E 4.130
		479A - 491E 3.091
		479A - 492E 4.422
		479A - 493E 3.445
		479A - 494E 4.179
		529A - 488E 4.670
		709A - 484E 4.758

		713A - 483E 2.737 713A - 484E 2.632 713A - 485E 4.900 716A - 483E 4.886 720A - 480E 4.178 721A - 477E 4.184 721A - 480E 3.496

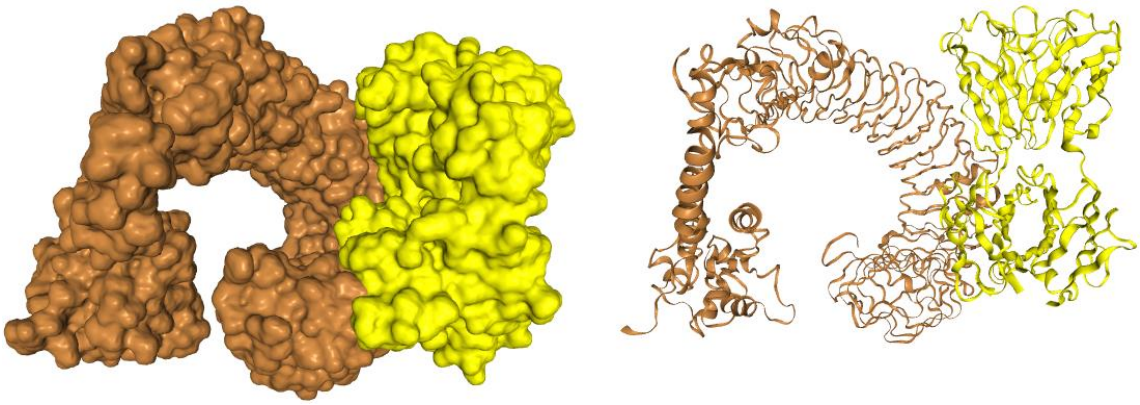

Figure-5: IL-6 with Chimeric ScFv McAB.

Table-8: Docking Score of IL-6 with Chimeric ScFv McAB.

Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Docking Score	-359.25	-346.07	-331.97	-328.49	-328.30	-328.30	-323.58	-316.59	-310.62	-305.55
Ligand RMSD (Å)	241.72	228.70	240.55	222.02	219.26	258.45	216.32	241.01	237.79	214.99

Table-9: Amino Acids Interaction of IL-6 with Chimeric ScFv McAB.

Receptor interface residue(s):	Ligand interface residue(s):	Receptor-ligand interface residue pair(s):
TYR 33A 3.210	GLY 8H 3.456	33A - 161H 3.210
ARG 34A 2.045	GLY 9H 3.328	34A - 160H 2.910
CYS 36A 3.207	GLY 10H 3.978	34A - 197H 4.662
PHE 55A 2.510	VAL 11H 3.044	34A - 198H 2.045
ASN 56A 3.685	VAL 12H 2.961	34A - 199H 4.138
TYR 57A 2.731	GLN 13H 2.679	34A - 200H 3.496
GLN 80A 2.297	ARG 16H 1.759	36A - 196H 4.267
TYR 81A 3.655	SER 17H 2.811	36A - 197H 4.848
THR 82A 1.679	LEU 18H 3.272	36A - 198H 3.207
SER 104A 3.367	ARG 19H 2.952	55A - 161H 3.816
LYS 106A 2.324	SER 21H 4.138	55A - 198H 3.836
TYR 108A 4.133	ASP 73H 4.369	55A - 200H 2.510
TYR 127A 3.284	LYS 76H 2.094	55A - 202H 4.187
PHE 128A 2.929	TYR 80H 4.138	55A - 213H 3.598
SER 132A 2.998	GLN 82H 3.512	56A - 215H 3.685
LYS 154A 3.192	SER 117H 4.849	57A - 195H 3.501
GLN 156A 3.505	SER 120H 4.142	57A - 196H 3.440
LEU 208A 2.924	THR 121H 2.924	57A - 198H 2.796
GLY 235A 4.719	LYS 122H 3.371	57A - 215H 2.731

TRP 238A 3.389	GLY 123H 4.388	57A - 217H 4.625
HIS 262A 3.117	PRO 124H 3.192	80A - 200H 2.297
HIS 263A 2.821	SER 125H 3.331	80A - 202H 4.966
ILE 264A 4.917	VAL 126H 4.133	80A - 213H 2.904
MET 265A 2.679	PRO 128H 4.495	80A - 214H 4.213
GLY 266A 4.563	ASN 160H 2.910	80A - 215H 2.990
PHE 271A 4.150	SER 161H 3.210	81A - 215H 3.655
HIS 272A 1.759	SER 178H 3.389	82A - 215H 1.679
ASN 273A 2.957	GLY 195H 3.501	82A - 217H 2.595
HIS 297A 3.734	THR 196H 3.440	104A - 213H 3.367
PHE 299A 3.044	GLN 197H 4.662	106A - 128H 4.495
PHE 301A 2.811	THR 198H 2.045	106A - 214H 3.487
LYS 323A 3.272	TYR 199H 4.138	106A - 124L 2.324
ASN 325A 2.952	ILE 200H 2.297	108A - 123L 4.584
LEU 348A 4.068	ASN 202H 4.187	108A - 127L 4.133
GLY 349A 3.115	PRO 207H 4.848	127A - 210H 3.995
GLU 350A 2.094	ASN 209H 3.734	127A - 211H 4.100
ALA 373A 3.512	THR 210H 3.995	127A - 212H 3.284
	LYS 211H 4.100	128A - 126H 4.133
	VAL 212H 3.284	128A - 214H 2.929
	ASP 213H 2.904	128A - 124L 4.525
	LYS 214H 2.929	132A - 127L 2.998
	ARG 215H 1.679	154A - 123H 4.652
	GLU 217H 2.595	154A - 124H 3.192
	ASP 123L 4.584	154A - 125H 3.331
	GLU 124L 2.324	154A - 126H 4.564
	LYS 127L 2.998	156A - 128L 3.505
	SER 128L 3.505	208A - 120H 4.142
		208A - 121H 2.924
		208A - 122H 3.371
		208A - 123H 4.388
		235A - 120H 4.719
		238A - 178H 3.389
		262A - 120H 4.377
		262A - 121H 3.117
		262A - 207H 4.848
		263A - 13H 2.821
		263A - 16H 4.634
		264A - 13H 4.917
		265A - 11H 3.459
		265A - 12H 2.961
		265A - 13H 2.679
		265A - 16H 3.317
		265A - 117H 4.849
		266A - 16H 4.563
		271A - 16H 4.150
		272A - 13H 4.444
		272A - 16H 1.759
		273A - 13H 2.976
		273A - 16H 2.957
		297A - 209H 3.734
		299A - 11H 3.044

		299A - 121H 3.836 301A - 17H 2.811 301A - 18H 3.291 301A - 19H 2.963 301A - 82H 3.512 323A - 8H 3.456 323A - 9H 3.328 323A - 10H 3.978 323A - 11H 4.498 323A - 18H 3.272 325A - 19H 2.952 348A - 19H 4.068 348A - 21H 4.138 349A - 19H 3.115 350A - 19H 4.313 350A - 73H 4.369 350A - 76H 2.094 350A - 80H 4.138 373A - 76H 3.512

Table-9: Solubility and Physicochemical properties of Chimeric Flagelline Mediated Tocilizumab Monoclonal Antibody.

Pysico chemical Properties	**Logp**	**Refractivity**	**Polarizability**	**Mass**	**Netcharge**
Monoclonal Antibody Complex	-448.10	6033.04 a3	2535.57A3	39705.77amu	0.20e

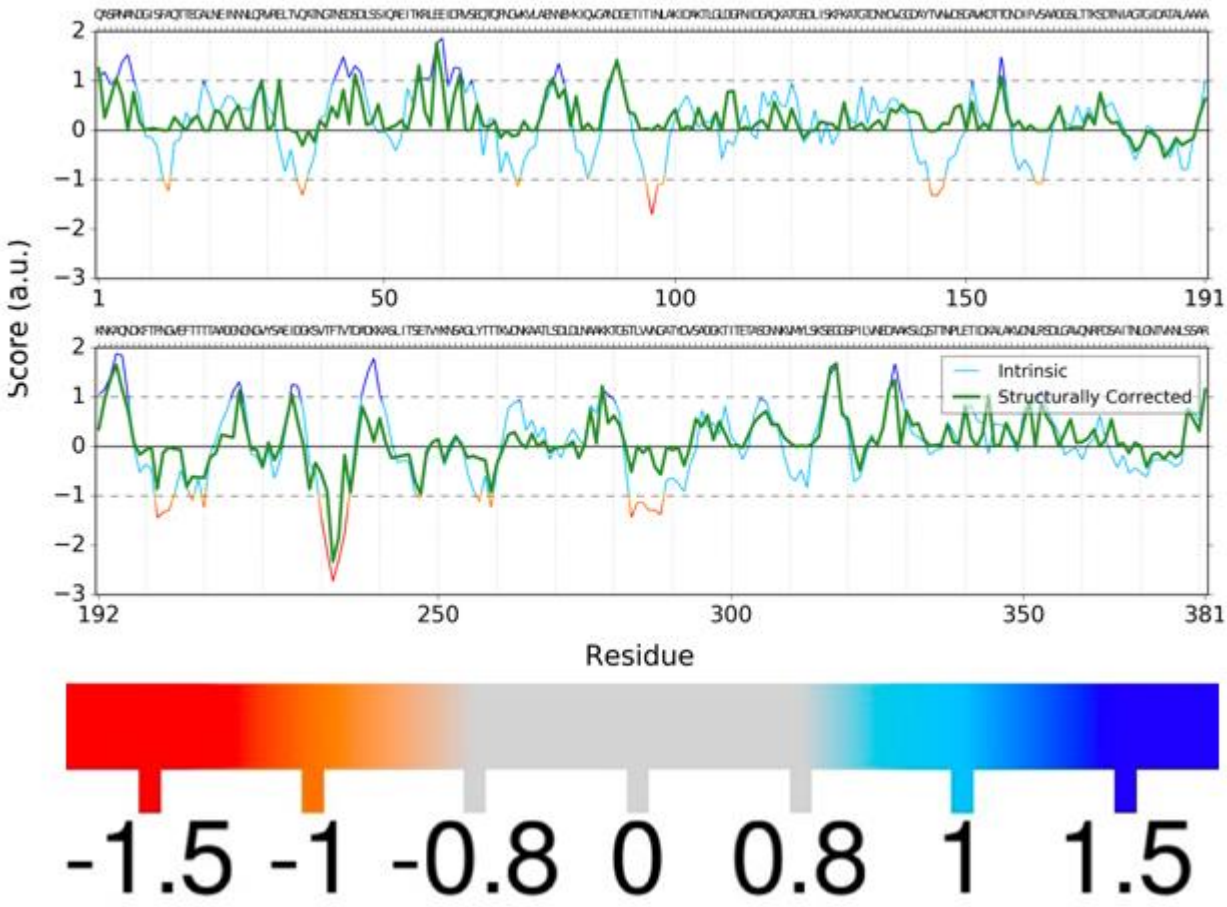

Figure-6: Shown The Physicochemical Properties and Solubility of Chimeric Monoclonal Antibody: Residue solubility range by color; Blue indicating highly soluble, and red indicating as poorly soluble

Molecular dynamics studies of Stability of structure:

The energy minimization & molecular dynamic study were analyzed by HyperChem. The data indicated that the E-Potential energy of the thermodynamic **of a Chimeric flagellin mediated Tocilizumab Monoclonal Antibody complex** was 2.08E+08, indicating that the chimeric protein mRNA is stable. Since the chimeric construct contains a flagellin (FliC) that is a part of a bacterial genotoxin.

Table-10: Molecular Dynamics study Indicating Stability study Results

Ligand	Time	E kinetic energy	E potential energy	E Tot	Temp
Chimeric flagellin mediated Tocilizumab Monoclonal Antibody complex	0.1 ps	7.01E+8 kcal	2.08E+08 kcal	9.09E+08	4.26E+07

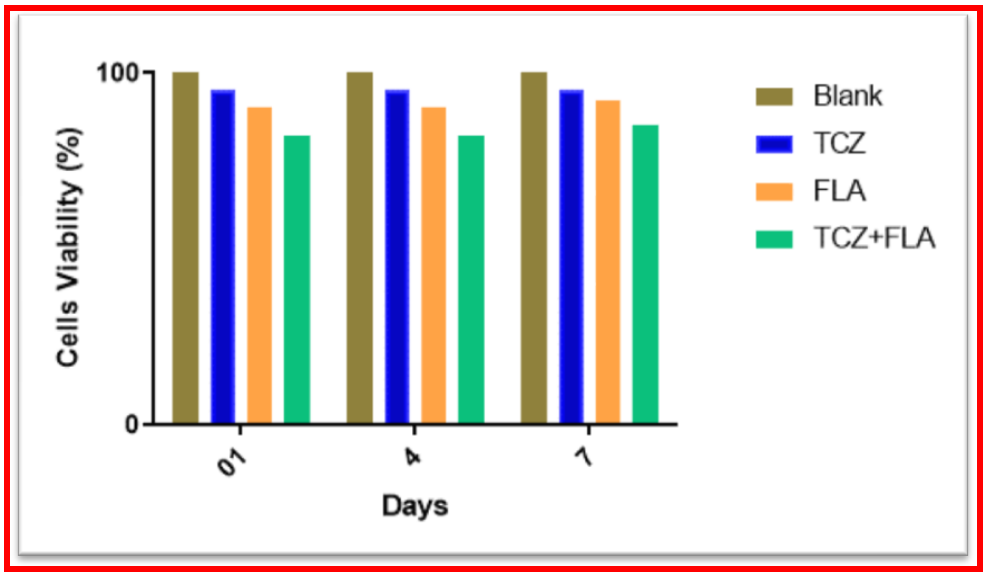

Figure-7: Shown MTT Viability assay of Fibroblasts in RA co-cultures after concentration-dependent incubation [10, 25, 50 and 100 ug/ml] with TCZ (Tocilizumab), Flagelline (FLAGLN) and Co-adminstration of TCZ + FLAGLN for 24 h on Day-1, 4 and 7-Days. Graphs show mean of n = 10 independent experiments for Fibroblasts in RA co-cultures. The viability of fibroblasts in RA co-cultures was dose dependently reduced after incubation with FLAGLN and Co-adminstration of TCZ + FLAGLN after 24 h. The combination (C0-administration) of TCZ+FLAGLN had shown the inhibition effect of Fibroblasts in RA Co-cultures.

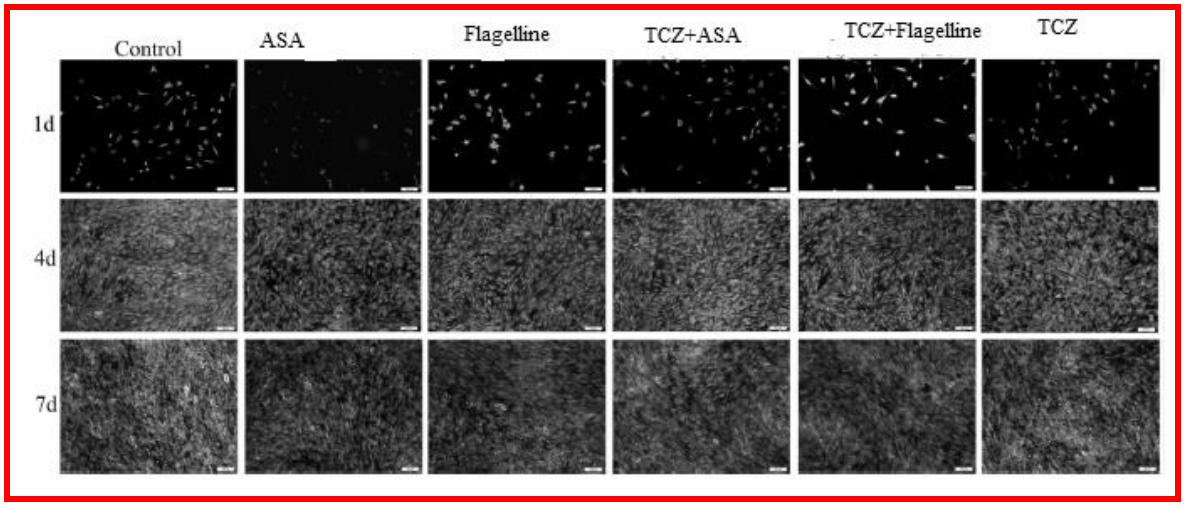

Figure-8: Shown the Cellular viability of images of AO/EB Staining of chondrocytes incubated with different samples such as controle, Acetyl Salicylic Acid (ASA as a STD), Flagelline, TCZ, TCZ+Flagelline and TCZ for 1 day, 4 days, and 7 days. (Scale bar = 10 μm).

Discussion:

When the immune system attacks the body's own tissues, it is known as autoimmunity and can lead to tissue damage (autoimmunity). Approximately 80 autoimmune diseases are known at this time. While some are widespread and simple to diagnose, such as type 1 diabetes, multiple sclerosis, lupus, and rheumatoid arthritis, others are uncommon and difficult. An estimated 700 million people worldwide, or close to 10% of the world's population, suffer with autoimmune disorders of varied severity. Tocilizumab (TCZ), is a recombinant humanized anti-interleukin-6 receptor (IL-6R) monoclonal antibody which has a main use in the treatment of rheumatoid arthritis, systemic juvenile idiopathic arthritis (sJIA) and polyarticular juvenile idiopathic arthritis (pJIA). Using tocilizumab injection **may decrease our ability to fight infection from bacteria, viruses, and fungi** and increase the risk that will get a serious or life-threatening infection that may spread through the body.

Overcome this limitations, the present work were focused for **Design and Development of a Chimeric Flagellin-mediated Tocilizumab Monoclonal Antibody for Autoimmune Disorders such as the** treatment of rheumatoid arthritis, systemic juvenile idiopathic arthritis (sJIA) and polyarticular juvenile idiopathic arthritis (pJIA).

Flagellin is a subunit protein of the flagellum, a whip-like appendage that enables bacterial motility. Traditionally, flagellin was viewed as a virulence factor that contributes to the adhesion and invasion of host cells, but now it has emerged as a potent immune activator, shaping both the innate and adaptive arms of immunity during microbial infections. In this work, we were conjugated the bacterial flagellin to the specific **Tocilizumab between heavy and light chain via specific linker then we get a** chimeric Single chain fragment variable that will interact both the innate and adaptive arms of immunity during infections and activate immune system and the role in flagellin as an adjuvant and immunomodulators.

Toll-like receptor 5 (TLR5) is a pattern recognition receptor, and Flagellin is its pathogen associated molecular pattern (PAMP), which stimulates host defense in a variety of organisms, including plants, insects, and mammals. This adjuvant property of Flagellin has been exploited by fusing polypeptides to Flagellin to render them antigenic. FliC is a unique PAMP because it harbors an antigenic hypervariable region, and a conserved domain which is involved in TLR5-dependent tumor regression and systemic, mucosal pro-inflammatory, and adjuvant activities. The Flagellin from Escherichia coli are the known paradigms for studies on flagellum structure-function, immunity, and TLR-5 signalling.

Under this exceeding background, the present investigation were designed to chimeric-ScFv constructs **(Shown Table-1 and Figure-1)** comprised of Tocilizumab light chain connected with Tocilizumab Heavy chain via a flexible linker like GGGGSGGGGSGGGGS, as a targeting molecule of Single chain variable fragments. The length and amino acid composition of the peptide linker is important in maintaining the scFv structure and stability. Typically, due to its flexibility, the linker peptide is about 3.5 nm (35°A) in length and contains hydrophobic amino acids such as glycine and serine residues. Furthered the bacterial flagellar antigens FliC (from Escherichia coli) which were conjugated via specific flexible linker RGRR (It's a linker derived from furin protease recognition site) finally we obtain a Chimeric ScFv. A 3D-homology model for this chimeric protein were constructed and its structure, stability, solubility and binding to IL-6 and TLR-5 were predicted via protein-protein docking which was shown good interactions were **shown on Table-1 to Table-9 and Figure-1 to Figure-6..**

The efficiency of this chimeric ScFv were depend on successful endocytosis, which is based on scFv and receptor interaction and internalization of the receptor-bound antigen-antibody This chimeric antibody/antigen is internalized via receptor-mediated endocytosis. After internalization, the complex can be sorted in early endosomes or in multivesicular bodies. Finally, Tocilizumab was recycled to the membrane and the Flagellin can go to Golgi. The cleavage of cytolethal distending toxin (CDT) is initiated by the furin protease of the Golgi system; then, it retrogrades to the endoplasmic reticulum (ER) and, ultimately, it is transported into the nucleus and leads to DNA damage. In accordance with this pathway and based on the fact that furin is enriched in the Golgi complex and acts as a protease for protein cleavage, a furin protease recognition site (RGRR amino acid sequence) was inserted between the Tocilizumab and the flagellin (FliC)-from Escherichia coli segment. It is predicted that when the scFv + flagellin (FliC)-from Escherichia coli, chimeric protein enters the Golgi, furin recognizes this site and breaks the peptide linker, which results in the scFv and Tocilizumab separation and the flagellin (FliC)-from Escherichia coli segment could reach the nucleus alone. This in-silico study outcome was shown the furin cleavage site is necessary for Flagellin activation in the Golgi apparatus and after scFv omission, the Flagellin can release to the cytoplasm. In accordance with the mentioned our Chimeric of antibody/antigen complex, when scFv cleaved by furin protease in Golgi, the conjugated flagellin (FliC)-from Escherichia coli) were transferred to the nucleus.

Under this proof of In-silico study, further the preliminary In-vitro cytotoxicity study attempt was on carried out and these major findings was shown on the MTT Viability assay of Fibroblasts in RA co-cultures after

concentration-dependent incubation [10, 25, 50 and 100 ug/ml] with TCZ (Tocilizumab), Flagelline (FLAGLN) and Co-adminstration of TCZ + FLAGLN for 24 hours. The results shown the mean of n = 10 independent experiments for Fibroblasts in RA co-cultures. The viability of fibroblasts in RA co-cultures was dose dependently reduced after incubation with FLAGLN and Co-adminstration of TCZ + FLAGLN after 24 h. The combination (C0-administration) of TCZ+FLAGLN had shown the good inhibition effect of Fibroblasts in RA Co-cultures on 10µg/ml. As a same manner, the Cellular viability of AO/EB Staining of chondrocytes incubated with different samples such as controle, Acetyl Salicylic Acid (ASA as a STD), Flagelline, TCZ, TCZ+Flagelline and TCZ for 1 day, 4 days, and 7 days. (Scale bar = 10 µm). The results shown the TCZ+Flagelline co-administration was shown the good inhibition effect of chondrocytes at 10µg/ml **was shown on Figure-7 to Figure-8.**

Conclusion:

Our findings suggest that the Flagelline mediated Tocilizumab monoclonal antibody, which was created by in-silico methods using chimeric ScFv, has good solubility, stability, and agonist and antagonist effects on TLR-5 and IL-6 respectivelly, based on binding and activation. The preliminary cytotoxicity study indicating the most active inhibition effects on fibroblast and chondrocytes with Co-adminstration of TCZ+Flagelline. Hence, the focus of future work will be based on the development, cloning, protein purification, characterization, and assessment of Chimeric flagelline mediated Tocilizumab monoclonal antibody for the treatment autoimmune disorders.

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