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Therapeutic Targeting of the IL-6/IL-17 Amplifier Loop in Fibroblasts: Translational Insights from Chronic Antibody-Mediated Rejection in Kidney Transplant Recipients

Mantabya K. Singh[1], Mohit Rai[2], Harshit Singh[1], Vikas Agarwal[2]
***Narayan Prasad**[1],

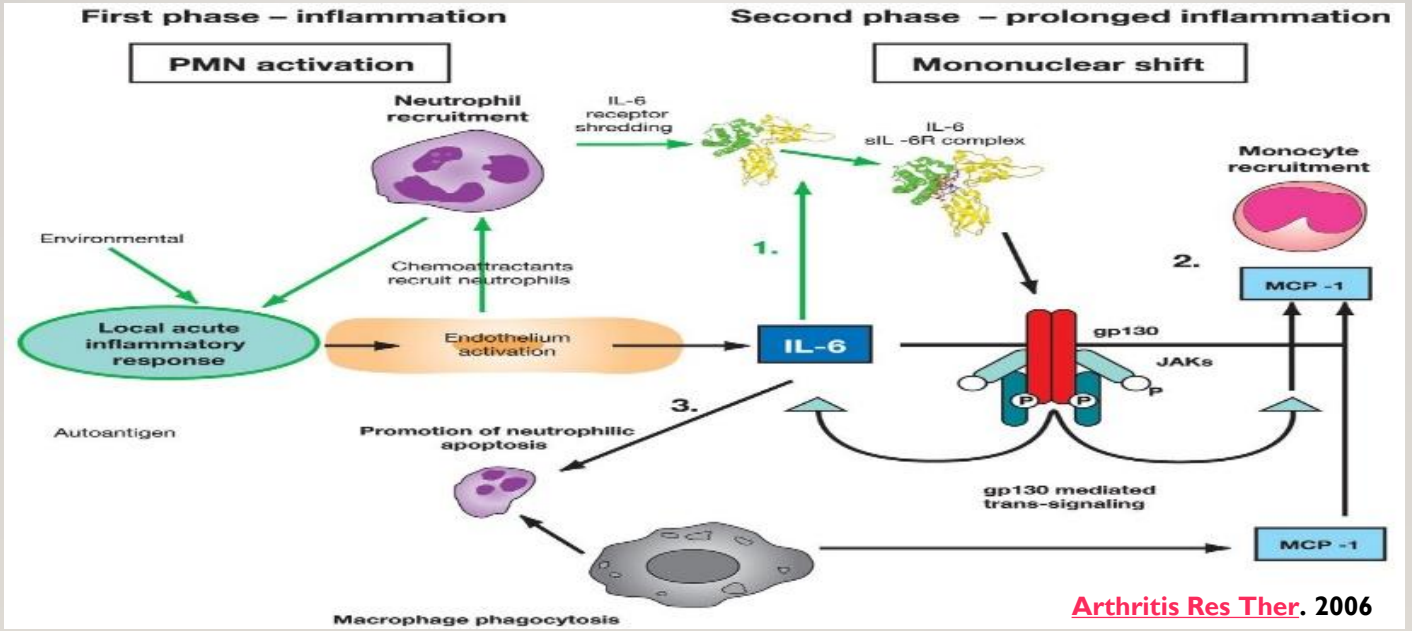
[1]Department of Nephrology and [2]Department of Clinical immunology

Sanjay Gandhi Postgraduate Institute Of Medical Sciences, Lucknow, India

INTRODUCTION

❖ Chronic antibody mediated rejection (CABMR) remains a major hurdle in long term Graft survival and management of CABMR is a challenging.

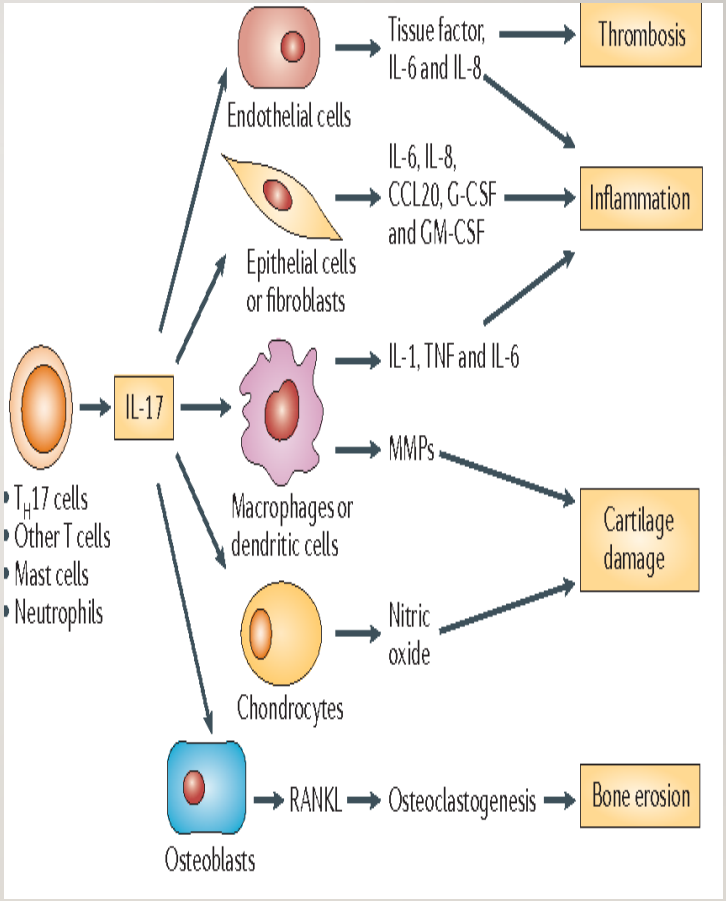
❖ IL-6 is a most important cytokine, plays central role in the development of chronic inflammation



Arthritis Res Ther. 2006

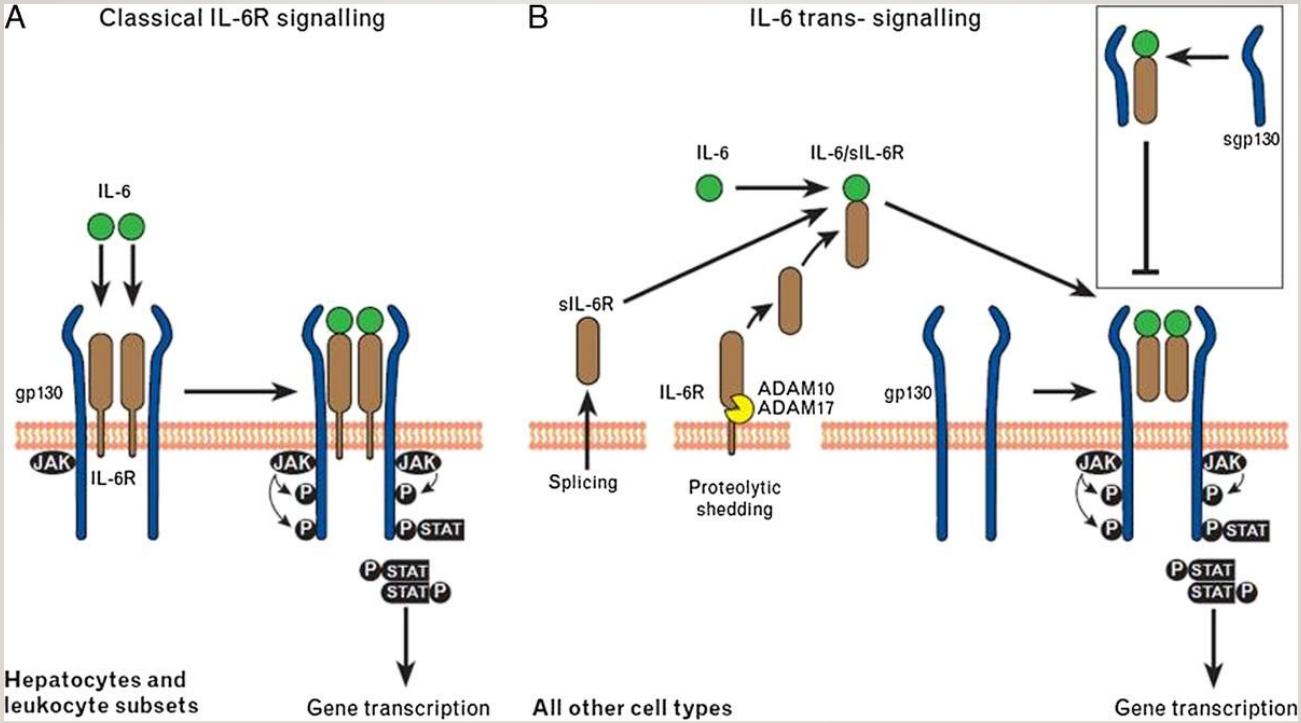
INTRODUCTION

- IL-17 has also been involved in the pathogenesis of infections, autoimmune and allergic disorders and play important role in allograft rejection.
- IL-17 activates inflammatory, endothelial, and epithelial cells and induces a variety of pro-inflammatory cytokines, chemokines and adhesion molecules.
- Excessive/inappropriate IL-17 production and/or Th17 cell activation have been reported to be involved in the development of chronic inflammatory diseases.
- IL-17-producing cells and enhanced IL-17 mRNA expression have been observed in human kidney transplants rejection.



Miossec, Pierre and Jay K. Kolls. "Targeting IL-17 and TH17 cells in chronic inflammation." Nature Reviews Drug Discovery 11 (2012): 763-776.

IL-6 Signalling during Chronic Inflammation

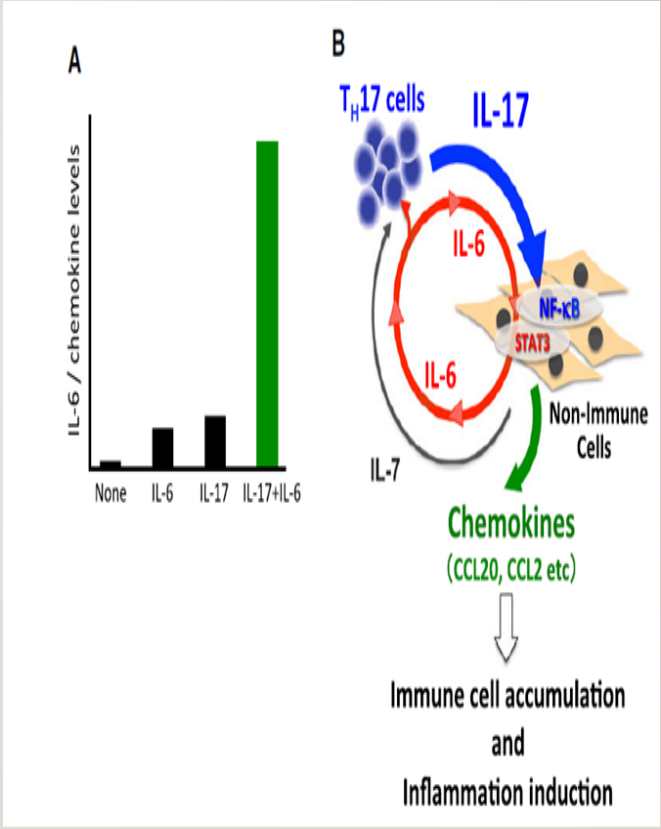


Jordan, Stanley C. MD et al, Transplantation: January 2017

IL-6 Amplifier Loop

- Previously we believed that alloreactive T-cell and de-novo DSA responsible for late term graft loss but resent study suggested that not only immune cell, non-immune cells like fibroblast also plays important role in chronic inflammation and allograft rejection via IL-6 amplifier loop (IL-6+IL-17).

- The interaction between non-immune tissues/cells and the immune system plays a critical role in chronic inflammation and late graft rejection.



Daisuke Kamimura , Masaaki Murakami
Front. Neurosci., 29 October 2013

Objectives

1. To study the effect of IL-6 + IL-17 on the secretion of IL-6 in the culture supernatant of fibroblast-derived from kidney biopsy of CABMR patients.
2. To elucidate the effect of Anti-IL-6 (Tocilizumab) and Anti-IL-17 on the secretion of IL-6 from the fibroblast derived from kidney tissue of CABMR patients.
3. To elucidate the Pro-inflammatory pathways leading to increased IL-6 secretion by induction of amplifier loop.

Results

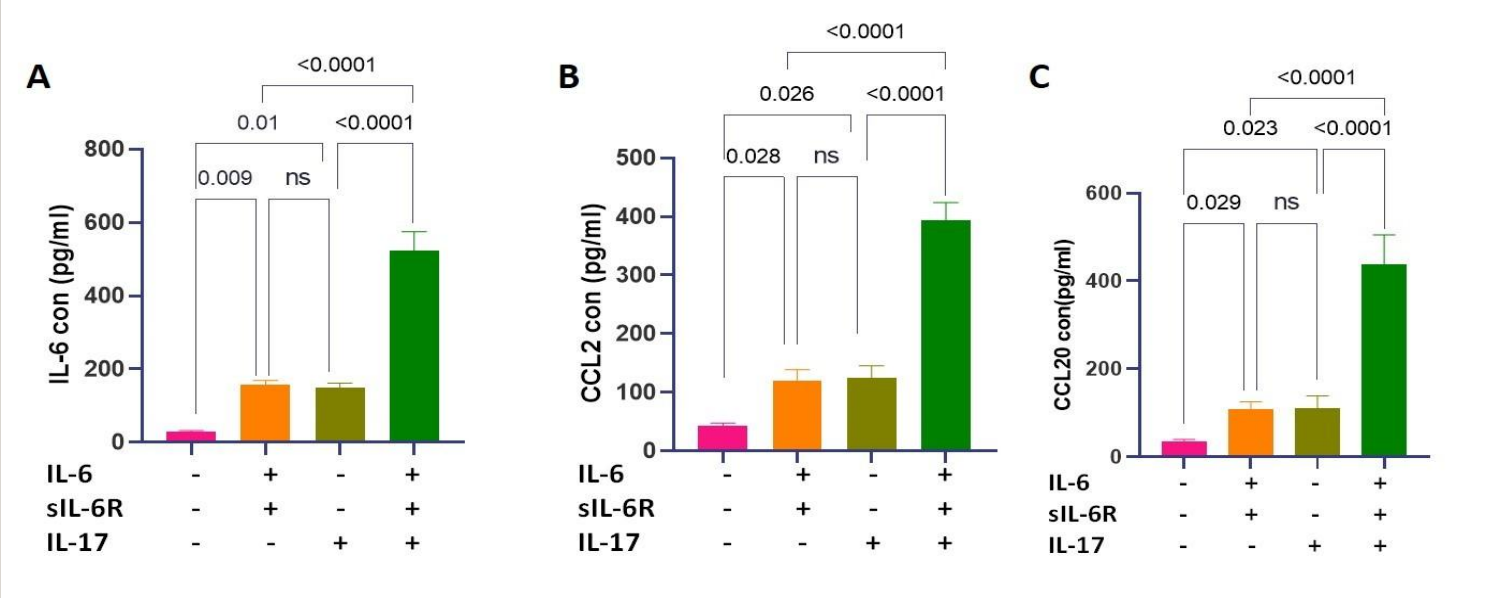
Demographic and clinical characteristics of CABMR patients

Demographic and Clinical Details	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Gender (M/F)	M	M	M	F	M	M
Age (Years)	43	66	58	46	37	42
ABO-c/ ABO-i	ABO-c	ABO-c	ABO-c	ABO-c	ABO-c	ABO-c
Post Transplant duration (months)	58	65	95	13	21	82
Hemoglobin (g/dl)	8.9	12.6	6.8	12.9	11.8	11.2
Tac level (ng/ml)	3.5	5.1	14.2	12	4.9	5.1
Serum Creatinine (mg/dl)	3.47	2.77	4.46	3.1	4.03	2.74
eGFR (mL/min/1.73 m2) (CKD-EPI Creatinine Equation)	20.4	12.8	24.1	27.6	17.8	47.1
BUN (mg/dl)	52.7	37.5	52.3	56.4	72.5	32.14
S. Uric Acid (mg/dl)	7.95	7.4	8.3	17.2	13.14	7.0
24 hr. Urine Protein/ Cr ratio	1.86	3.46	3.35	4.19	3.93	4.167
Induction regimen						
Induction	No induction	Basiliximab	Basiliximab	ATG	No induction	No induction
Maintenance Immunosuppression	MMF+ Pred+ Tacrolimus	MMF+ Pred+ Tacrolimus	MMF+ Pred+ Tacrolimus	MMF+ Pred+ Tacrolimus	MMF+ Pred+ Tacrolimus	MMF+ Pred+ Tacrolimus

Histological grade scoring and DSA's mean fluorescent intensity (MFI) value on SAB assays of CABMR patients following Banff classification criteria 2019

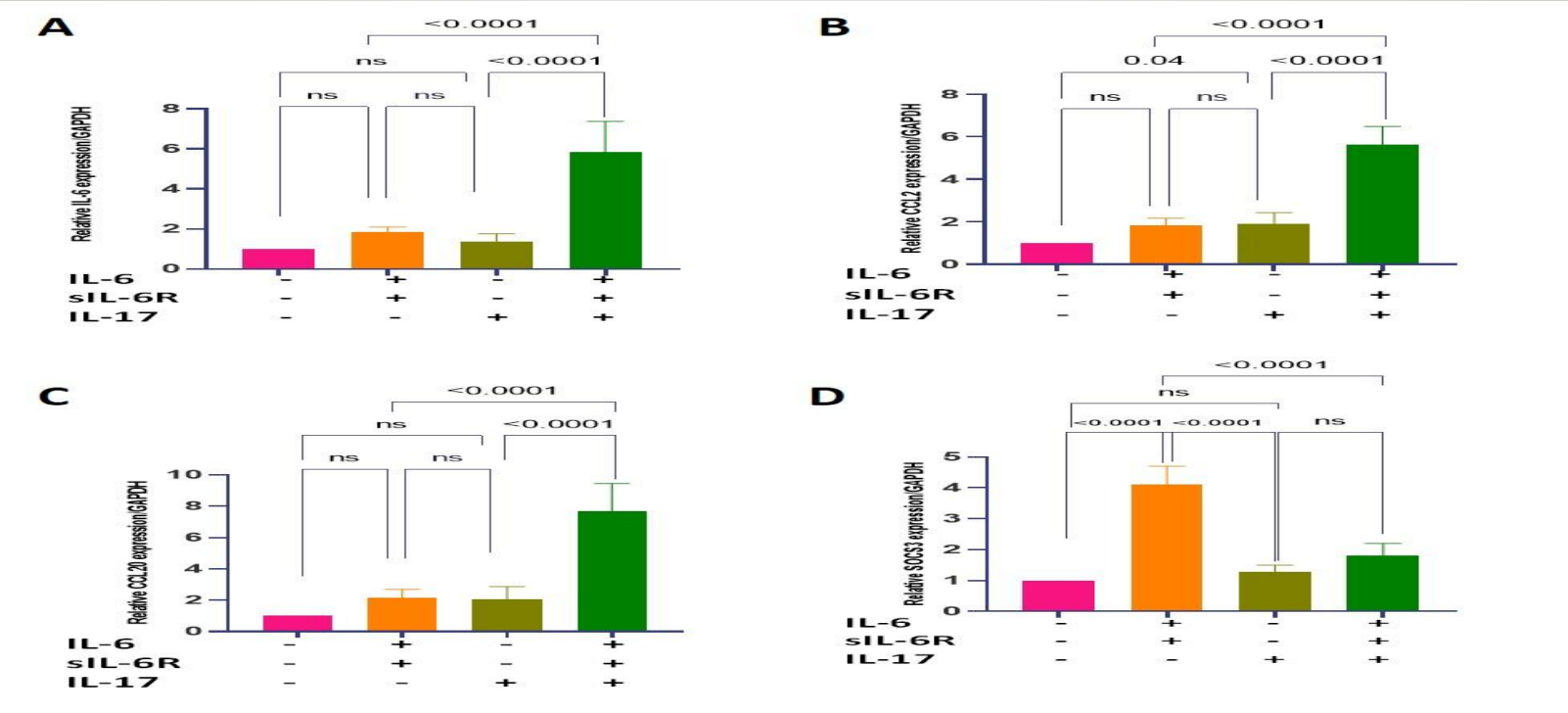
Characteristics	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Peritubular capillaritis	ptc 2	ptc 2	ptc 2	ptc 3	ptc 2	ptc 1
Glomerulitis	g 2	g 2	g 1	g 2	g 2	g 1
Tubulitis	t 0	t 1	t 0	t 0	t 1	t 1
Transplant Glomerulopathy(cg score)	cg3	cg3	cg2	cg3	cg1	cg1
Interstitial Inflammation	i 1	i1	i 2	i 1	i 1	i 1
Interstitial fibrosis (IF)	ci1	ci2	ci2	ci1	ci1	ci2
Tubular Atrophy (TA)	ct1	ct1	ct2	ct1	ct1	ct1
C4d staining	Positive	Positive	Positive	Positive	Positive	Positive
DSA(MFI)-SAB data						
Class-I	Positive (MFI- 4655)	Positive (MFI-11289)	Positive (MFI- 7039)	Positive (MFI- 7109)	Positive (MFI- 3184)	Positive (MFI- 1951)
Class-II	Positive (MFI-4327)	Positive (MFI- 12497)	Positive (MFI- 21703)	Positive (MFI- 15177)	Positive (MFI- 5489)	Positive (MFI- 2466)

Establishment of Amplifier loop in CABMR Patients



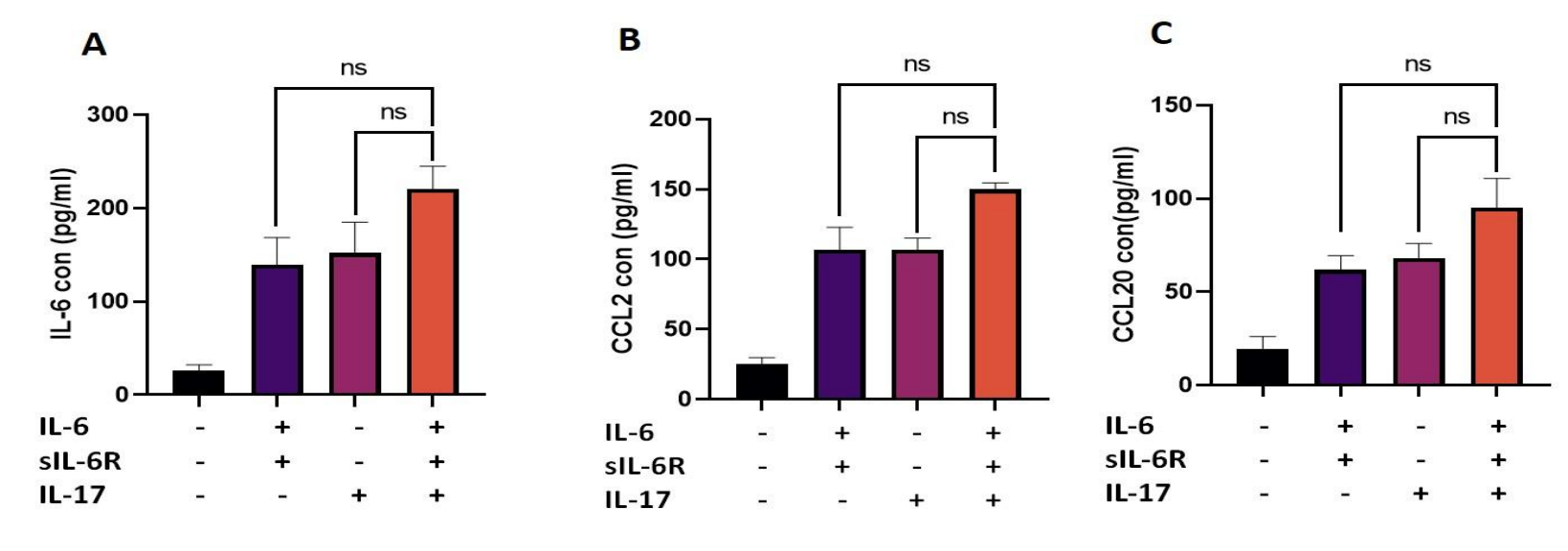
IL-6 amplifier loop activation in the presence of IL-6 and IL-17A triggered IL-6 expression in human Primary Renal Fibroblast cells. stimulated with IL-6 (20 ng/ml), sIL-6R (20 ng/ml), IL-17 (50 ng/ml) and a combination of IL-6+sIL-6R+IL-17 for 24, respectively. **(A)** IL-6, **(B)** CCL2 and **(C)** CCL20 levels were measured in culture supernatant by ELISA respectively. Error bars represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Experiments were performed at least two times; representative data are shown.

Establishment of Amplifier loop in CABMR Patients



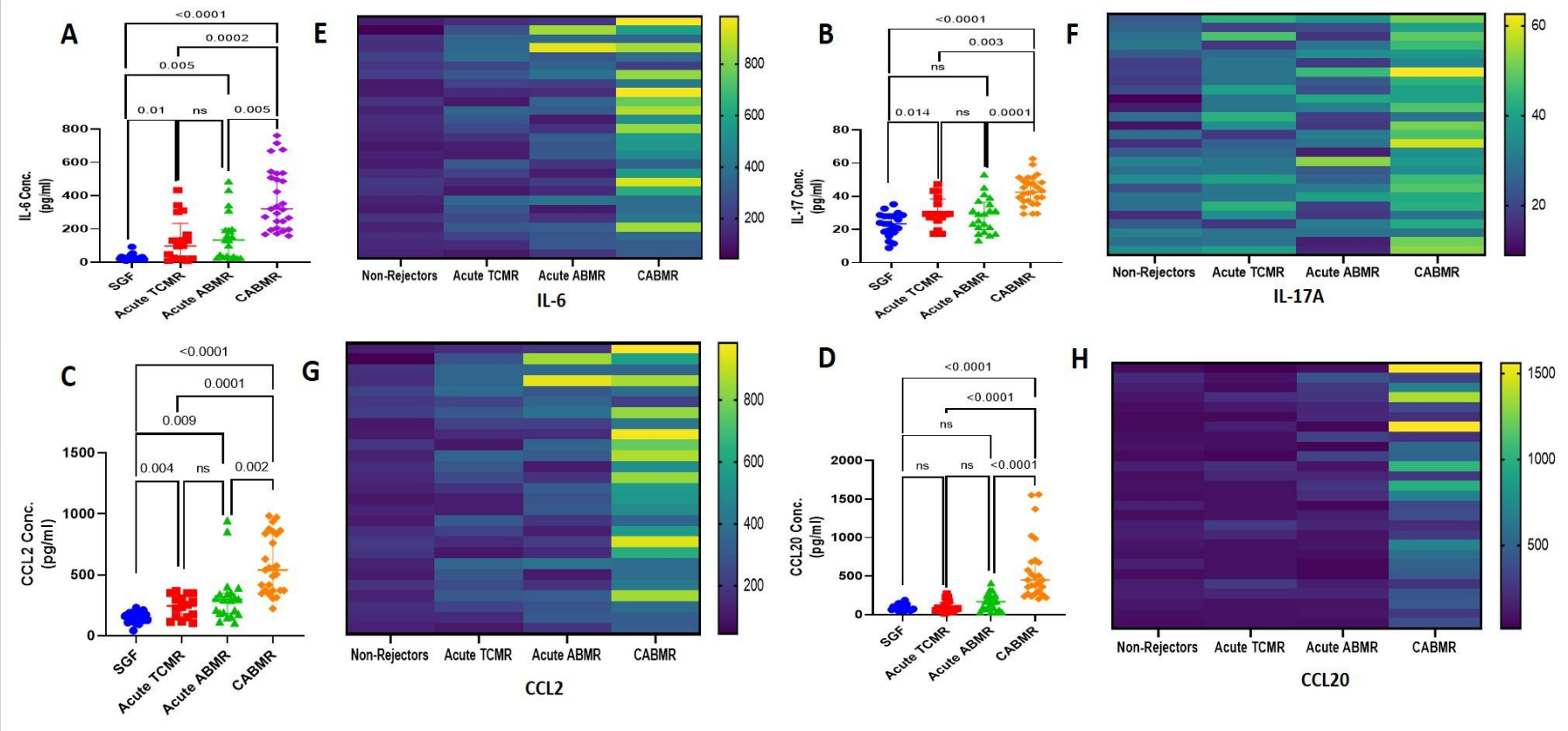
IL-6 amplifier loop activation in the presence of IL-6 and IL-17A triggered IL-6 expression in human Primary kidney Fibroblast cells. stimulated with IL-6 (50 ng/ml), sIL-6R (50 ng/ml), IL-17 (50 ng/ml) and a combination of IL-6+sIL-6R+IL-17 for 3 hour, respectively. mRNA expression was evaluated using real time PCR (Syber green) (A) IL-6, (B) CCL2 and (C) CCL20 and (D) SOCS3. Error bars represent the mean ± SEM. $P<0.05$, was significant, $P<0.0001$, was considered to be highly significant.

Establishment of Amplifier loop in Nephrectomy sample



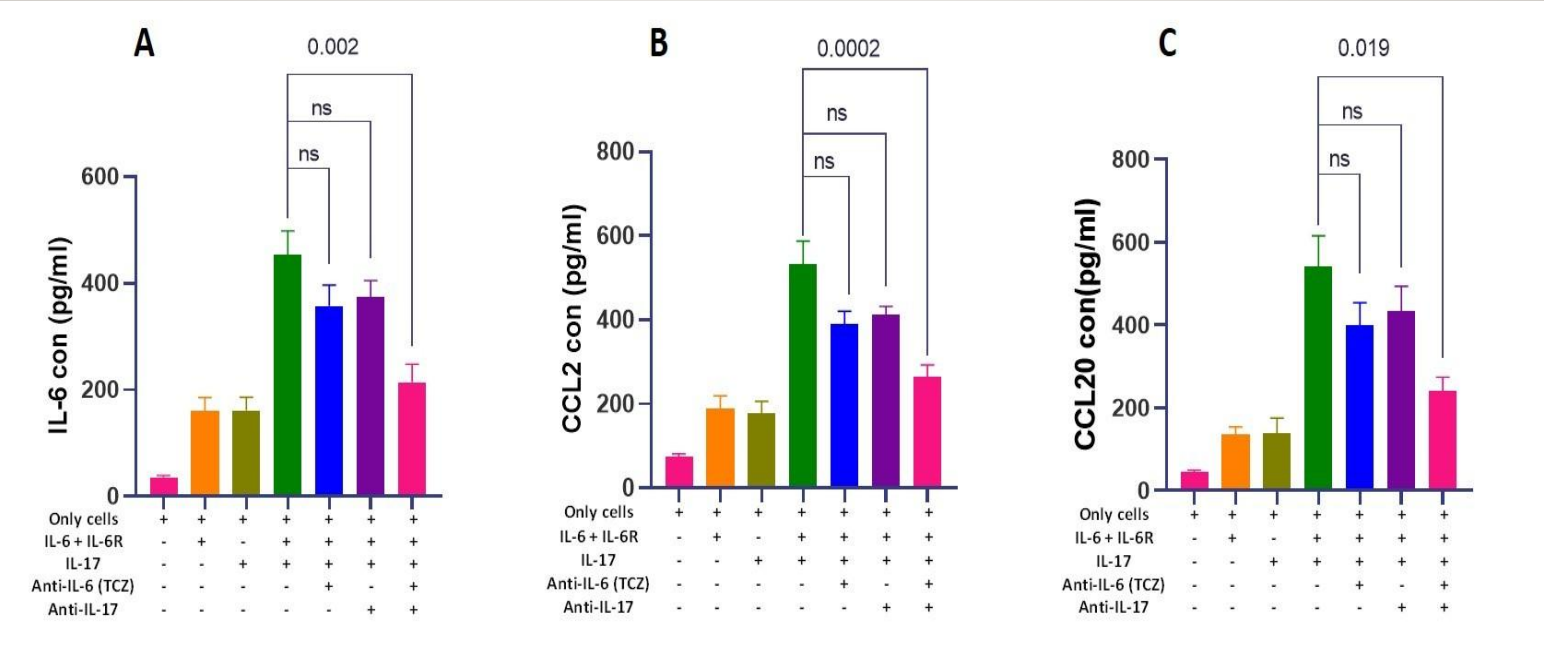
IL-6 amplifier loop not activated in the presence of IL-6 and IL-17A triggered IL-6 expression in human Primary Renal Fibroblast cells derived from Nephrectomy sample. stimulated with IL-6 (20 ng/ml), sIL-6R (20 ng/ml), IL-17 (50 ng/ml) and a combination of IL-6+sIL-6R+IL-17 for 24, respectively. IL-6, CCL2 and CCL20 levels were measured in culture supernatant by ELISA respectively. Error bars represent the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Experiments were performed at least three times; representative data are shown.

Serum cytokines and chemokines level (pg/ml) in patients of SGF, acute TCMR, acute ABMR and CABMR group



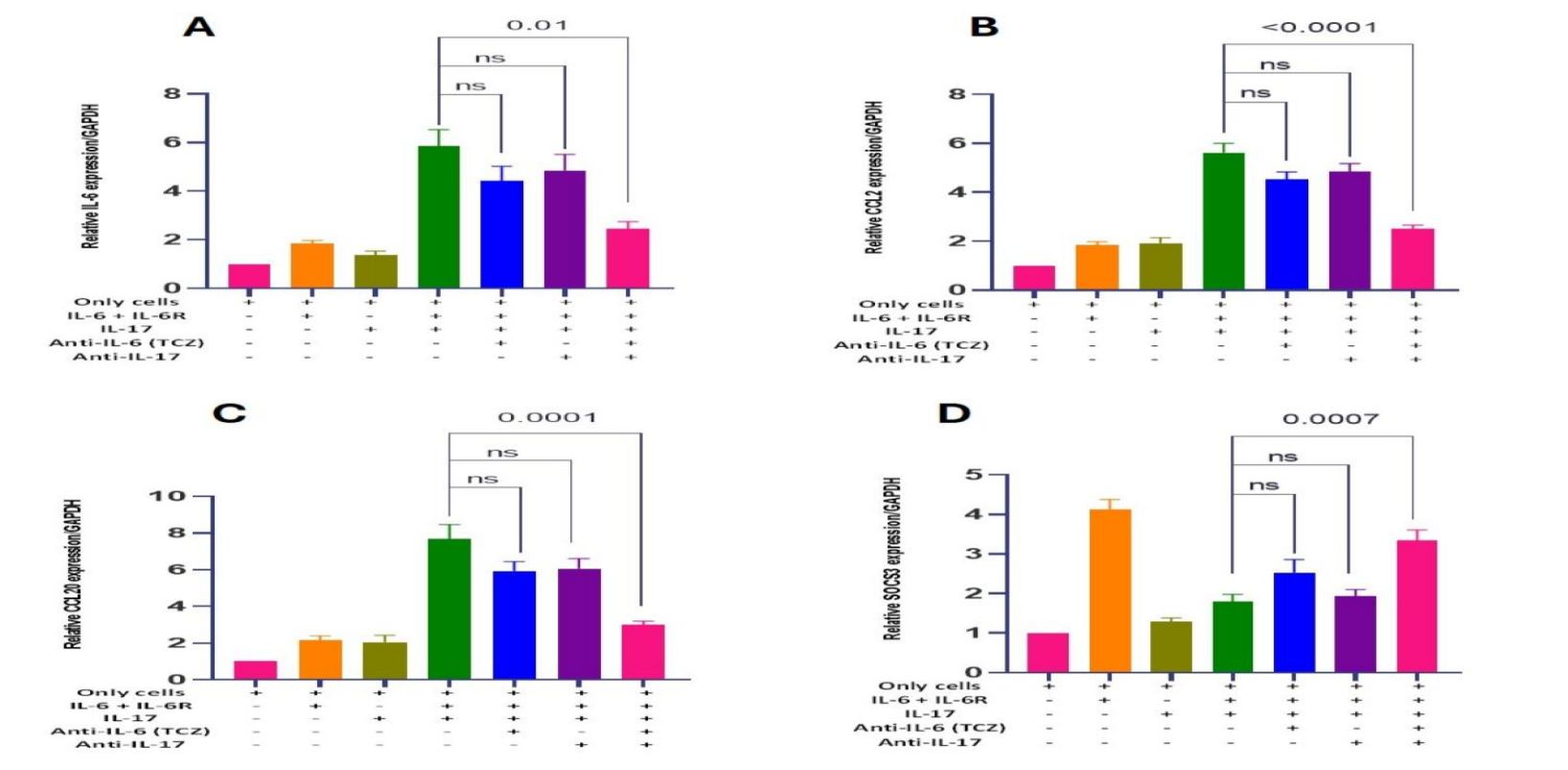
Serum cytokines and chemokines level (pg/ml) in patients of SGF, acute TCMR, acute ABMR and CABMR group measured by ELISA (A, B, C and D). Heat map showing the significant cytokine and chemokines profile changes for IL-6, IL-17, CCL2 and CCL20 (E, F, G, H) involved in Kidney transplant rejection patients. The representative data were shown in median with IQR. $P<0.05$, was significant, $P<0.0001$, was considered to be highly significant. IQR: Inter quartile range.

Effect of TCZ and Anti-IL-17 on Amplifier loop in culture supernatant



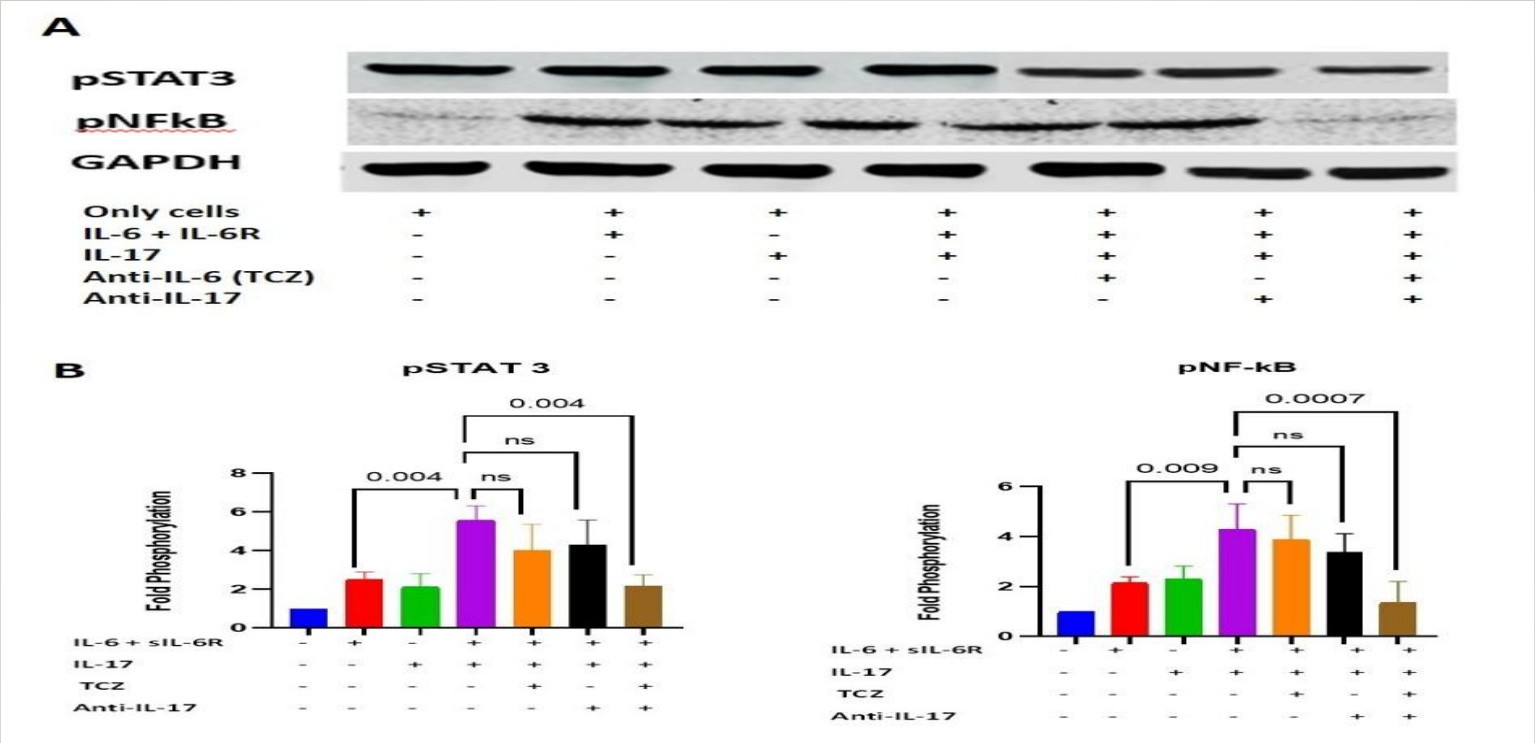
IL-6 amplifier activation is Attenuated in the presence of IL-6 and IL-17 inhibitor in human Primary Renal Fibroblast stimulated with IL-6 (20 ng/ml), sIL-6R (20 ng/ml), IL-17 (50 ng/ml) and a combination of IL-6+sIL-6R+IL-17 for 24, respectively. Anti-IL-6 (100ng/ml) and Anti-IL-17 (0.75 ng/ml) were added to the cells 30 min before cytokine stimulation **(A)** IL-6, **(B)** CCL2 and **(C)** CCL20 levels were measured in culture supernatant by ELISA respectively. Error bars represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Experiments were performed at least two times; representative data are shown.

Gene Expression Analysis at m-RNA level with TCZ and Anti IL-17 treatment

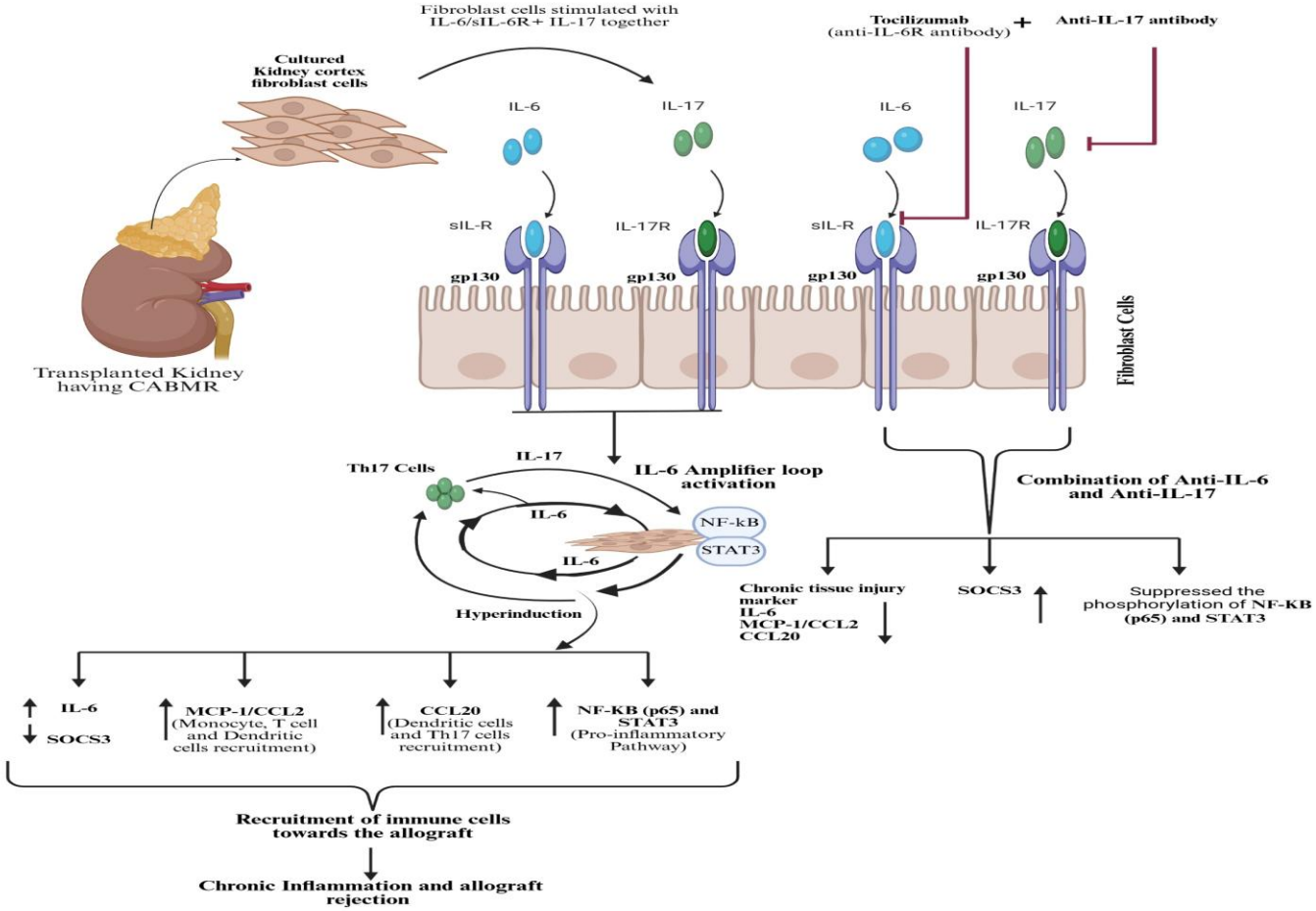


IL-6 amplifier activation in the presence of IL-6 and IL-17 in human Primary Renal Fibroblast stimulated with IL-6 (50 ng/ml), sIL-6R (50 ng/ml), IL-17 (50 ng/ml) and a combination of IL-6+sIL-6R+IL-17 for 3 hours, respectively. Anti-IL-6 (100ng/ml) and Anti-IL-17 (0.75 ng/ml) were added to the cells 30 min before cytokine stimulation, followed by **(A)** IL-6, **(B)** CCL2, **(C)** CCL20, and **(D)** SOCS3 gene expression by real-time PCR. Data are expressed as a fold change of expression relative to unstimulated samples and normalized to the GAPDH respectively. Error bars represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Experiments were performed at least two times; representative data are shown.

Amplifier loop activation enhances the NF-KB and STAT3 pathway



The IL-6 and IL-17 signal enhances the NF-kB (p65) and STAT3 signalling pathway. kidney fibroblast cells were stimulated with IL-6 (50 ng/ml), sIL-6R (50 ng/ml) and/or IL-17 (50 ng/ml) for 60 min. Anti-IL-6 (100ng/ml) and Anti-IL-17 (0.75 µg/ml) were added to the cells 30 min before cytokine stimulation. Whole cell lysate was prepared and analyzed by western blotting with anti-phospho-NF-kB, anti-phospho-STAT3 and loading control anti-GAPDH antibody. (A) Densiometric scan were normalized to naïve GAPDH levels. (B) Fold phosphorylation of NF-kB (p65) and STAT3 protein. Data were shown in mean ± SEM. $P<0.05$, was significant. TCZ: Tocilizumab.



Summary of the effect of Anti-IL-6 and Anti-IL-17 on Amplifier loop activation with IL-6/sIL-6R and IL-17 stimulation on kidney Fibroblasts. After allogenic transplantation, the non-immune cells like fibroblast triggers the IL-6 Amplifier loop activation via stimulation with IL-6/sIL-6R and IL-17 together. This cause the hyperactivation of IL-6, CCL2 and CCL20 via STAT3 and NF-kB (p65) signalling pathway that cause the accumulation of immune cells towards the allogenic graft, that promote chronic inflammation and chronic allograft rejection. Inhibition with anti-IL-6 and anti-IL-17 together supress the IL-6, CCL2 and CCL20 with decreased phosphorylation of STAT3 and NF-kB (p65) from the fibroblast cells that could possibly reduce the chronic inflammation and allograft rejection.

Conclusion

- We conclude that the fibroblasts from patients with CABMR may be the epicentre of IL-6/IL-17 amplifier loop activation.
- A combination of IL-6, sIL-6R, and IL-17 stimulation enhanced the quantity of IL-6, CCL2 and CCL20 in the culture supernatant and their mRNA expression after stimulating fibroblast.
- The SOCS3 gene was downregulated after amplifier loop activation, indicating prolonged signalling of IL-6.
- The phosphorylation of NF-kB (p65) and STAT3 protein was increased in the presence of IL-6, sIL-6R, and IL-17 from the fibroblast cells.
- Inhibition of IL-6 with Anti-IL-6 and IL-17 with Anti-IL-17 reduced tissue injury markers IL-6, MCP1, and CCL20 in culture supernatant as well as at mRNA level and simultaneously upregulate the SOCS3 gene.
- Combining IL-6 and IL-17 inhibitors suppressed the phosphorylation of STAT3 and NFkB proteins.
- Thus, our in vitro study suggests that inhibition of the IL-6 amplifier loop with a combination of anti-IL-6 and anti-IL-17 may be a therapeutic target in cases of CABMR, which constitutes future directions for a clinical trial.