Role of Damage associated-molecular patterns in chronic renal injury associated inflammation

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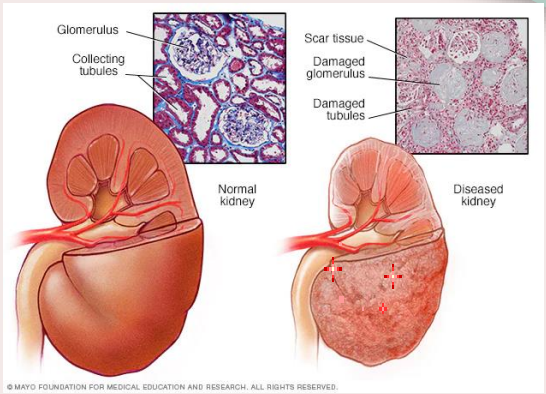
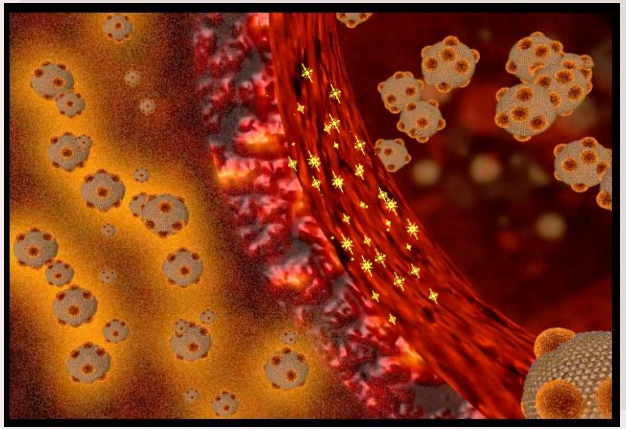
Chronic renal failure

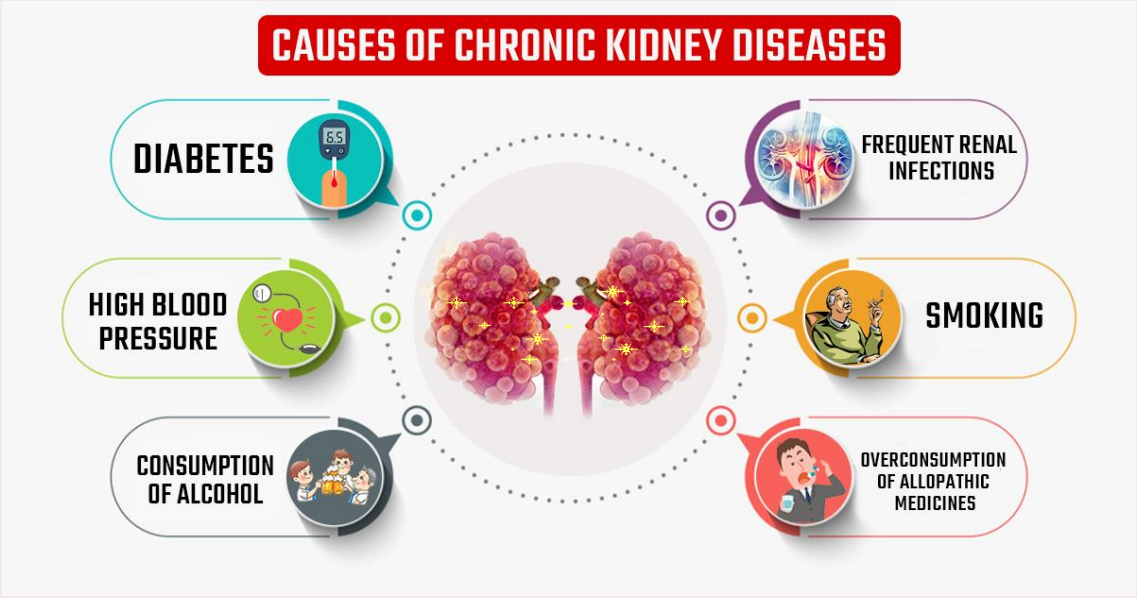
- Worldwide public health problem
- now recognized as a common condition associated with an increased risk of cardiovascular complications due to atherosclerotic changes





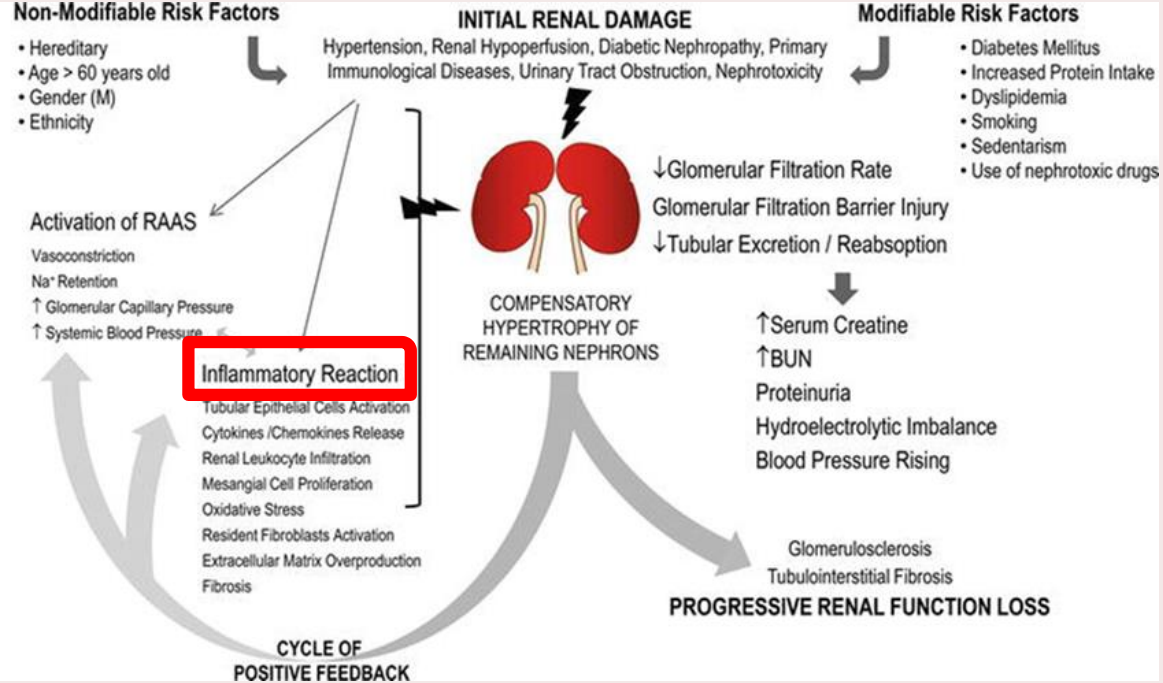
Main causes of CKD

- Diabetes and hypertension
- Autoimmune disorders, generally designated by Glomerulonephritis (GN)
- (IgA nephropathy
- renal vasculitis
- transplant rejection
- lupus nephritis
- postinfectious glomerulonephritis)



CKD

- Upon injury or pathogen invasion, the innate immune system acts as an immediate defense mechanism, to provide cytoprotective mechanisms for tissue healing.
- Activated immune-inflammatory cascade is one of the most important triggering factor
- Persistent or excessive immune system activation is harmful and results in permanent organ structural and function alterations.



Pathogen-Associated Molecular Patterns (PAMPs)

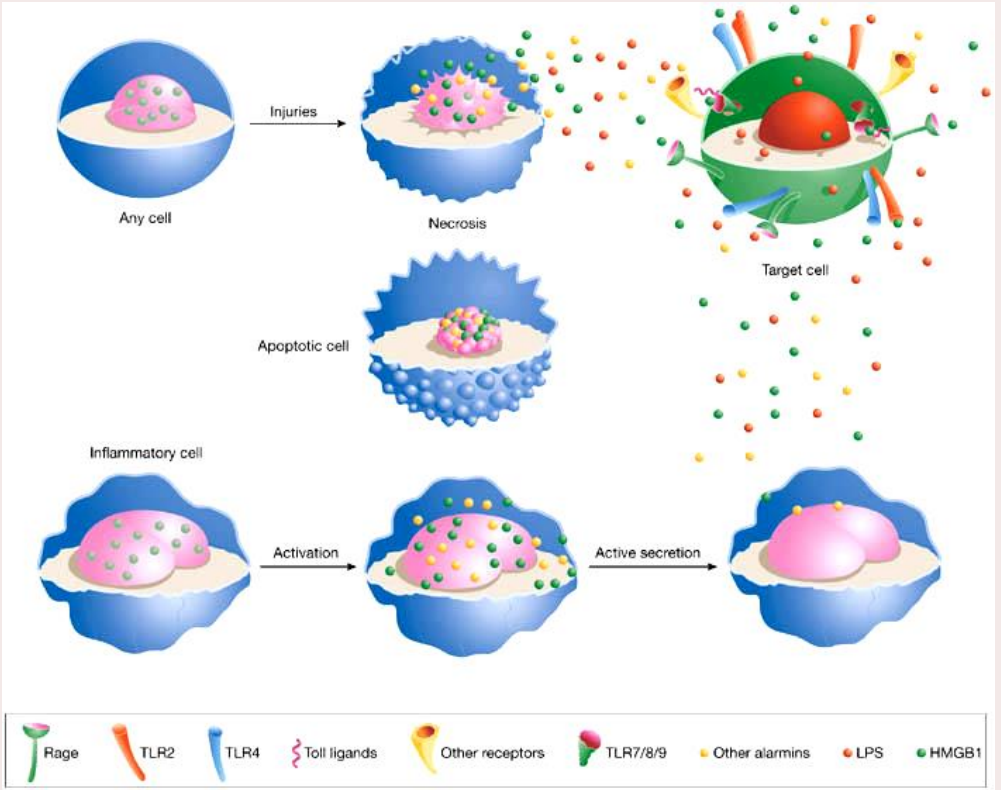
PAMPs are different structures shared by large groups of microorganisms, absolutely essential for the microbe's survival:

LPS: common component of gram-negative bacteria
teichoic acids: common component of gram-positive bacteria
double-stranded RNA: a structural signature of several groups of RNA viruses
bacterial DNA (unmethylated CpG sequences)

DAMP molecules, alarmins, endokines

Their family includes different molecules

The term DAMP denotes structurally diverse **multifunctional host molecules** that are rapidly released during infection or tissue damage with activating effects on receptor-expressing cells engaged in host defence and tissue repair.



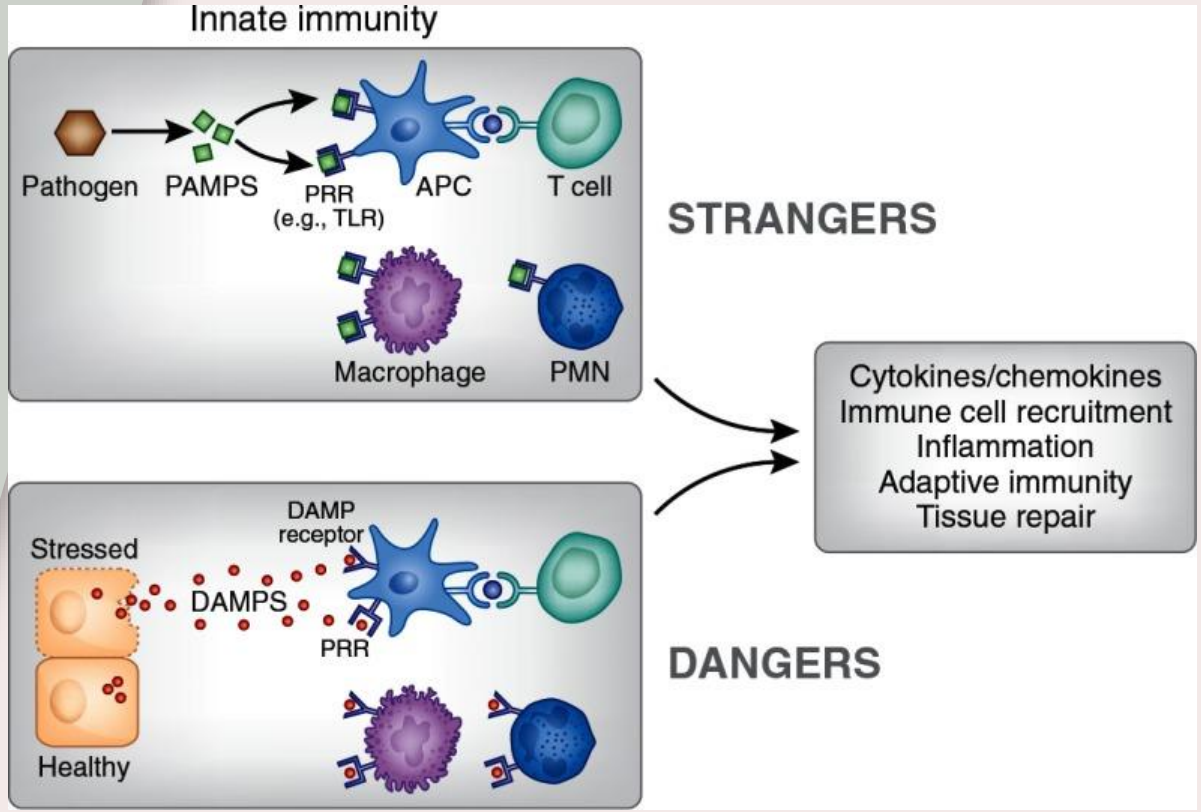
Damage-Associated Molecular Patterns (DAMPs)

Table 1 | Adjuvant and pro-inflammatory activity of intracellular DAMPs

DAMP	Adjuvant activity	Pro-inflammatory activity	Potential receptors
HMGB1	*In vivo*: adjuvant activity of purified molecule; adjuvant activity shown by selective depletion	*In vivo*: inflammation in response to liver injury blocked by neutralizing antibody; neutrophil recruitment induced by purified molecule	RAGE, TLR2*, TLR4
	In vitro: DC activation	*In vitro*: chemotaxis; cytokine induction	
Uric acid (MSU)	*In vivo*: adjuvant activity shown by injection of purified molecule and selective depletion	*In vivo*: gout induced by purified molecule; neutrophil recruitment induced by purified molecule	TLR2*, TLR4*, CD14
	In vitro: DC activation	*In vitro*: cytokine induction	
Chromatin, nucleosomes and DNA	*In vivo*: DC maturation induced by purified molecule	*In vivo*: neutrophil recruitment induced by purified molecule	TLR9 (with BCR or Fc receptor)
	In vitro: DC activation induced by chromatin–IgG complexes	*In vitro*: cytokine induction; B-cell activation induced by chromatin–IgG complexes	
HSPs	*In vivo*: tumour immunogenicity enhanced by overexpressed molecule or addition of purified molecule (HSP70); DC migration to lymph nodes induced by purified molecule (gp96)	*In vivo*: ND	CD14 (HSP70 and HSP60); CD91 (HSP70, HSP90, gp96 and calreticulin); scavenger receptors (HSP70, gp96 and calreticulin); TLR4 (HSP60); TLR2* and TLR4* (HSP60 and gp96); CD40 (HSP70)
	In vitro: DC maturation (gp96 and HSP70)	*In vitro*: cytokine induction (HSP60, HSP70, HSP90 and gp96)	
Adenosine and ATP	*In vivo*: exacerbation or abrogation of bronchial asthma by purified molecule or specific inhibition, respectively	*In vivo*: exacerbation of nephritis by purified molecule	P1, P2X and P2Y receptors (ATP); A1, A2A, A2B and A3 receptors (adenosine)
	In vitro: DC maturation	*In vitro*: chemotaxis	
Galectins	*In vivo*: ND	*In vivo*: monocyte recruitment induced by purified molecule	CD2 and others containing β-galactose
	In vitro: DC maturation	*In vitro*: chemotoxis	
Thioredoxin	ND	*In vivo*: chemotaxis induced by purified molecule	ND
		In vitro: chemotaxis	
S100 proteins	ND	*In vivo*: neutrophil recruitment induced by purified molecule	RAGE
		In vitro: chemotaxis; cytokine induction	
Cathelicidins	*In vitro*: DC maturation; DC activation induced by LL37–self-DNA complex	*In vitro*: chemotaxis	FPRL1
Defensins	*In vivo*: adjuvant activity by co-administration of purified molecule	*In vivo*: ND	CCR6 and TLR4
	In vitro: DC maturation	*In vitro*: chemotaxis	
N-formylated peptides	*In vivo*: ND	*In vivo*: neutrophil recruitment induced by purified molecule	FPR and FPRL1
	In vitro: DC chemotaxis	*In vitro*: chemotaxis	

DAMPs can be actively secreted by inflammatory cells and passively released by necrotic cells.

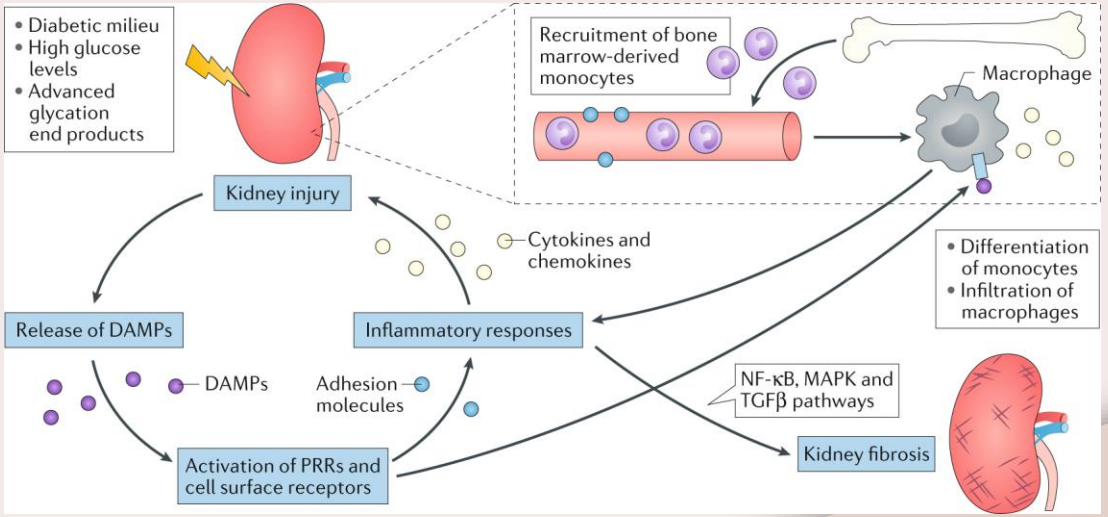
Strangers vs Dangers



“Danger hypothesis” postulates that the immune system is evolved to respond not only to the infective agents, but also to “non-physiological” cell death, damage or stress

Strangers vs Dangers

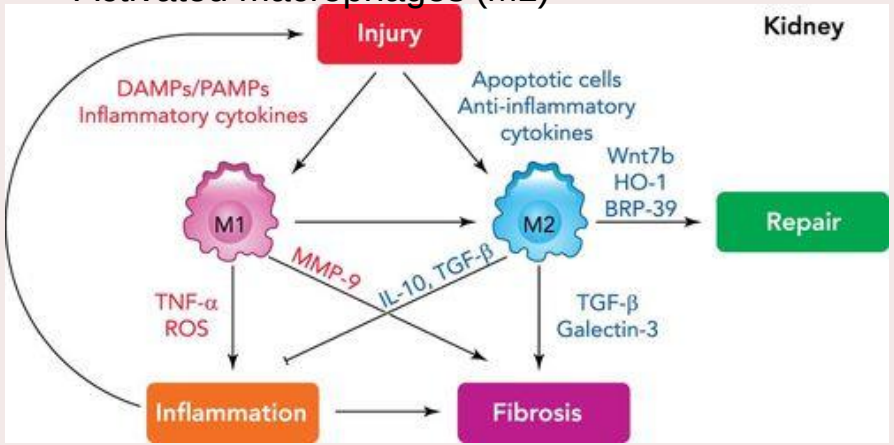
- Like PAMPs, DAMPs derived molecules may initiate strong inflammatory response, via the activation of antigen presenting cells (APC)
- DAMP induce production of pro-inflammatory cytokines (IL-1, TNF-α, IL-6, IL-8) and the upregulated expression of cell adhesion molecules (ICAM-1, VCAM-1)



Inflammation in CKD

- Macrophages are found in normal kidney
- In increased numbers in diseased kidney, act as key players in renal injury, inflammation, and fibrosis.
- The recruitment and differentiation of macrophages are crucial to all phases of renal injury, from triggering tissue injury to tissue repair.

Activated macrophages (M1)



Alternatively activated macrophages (M2), produced by Th2 cytokines such as IL-4 and IL-10

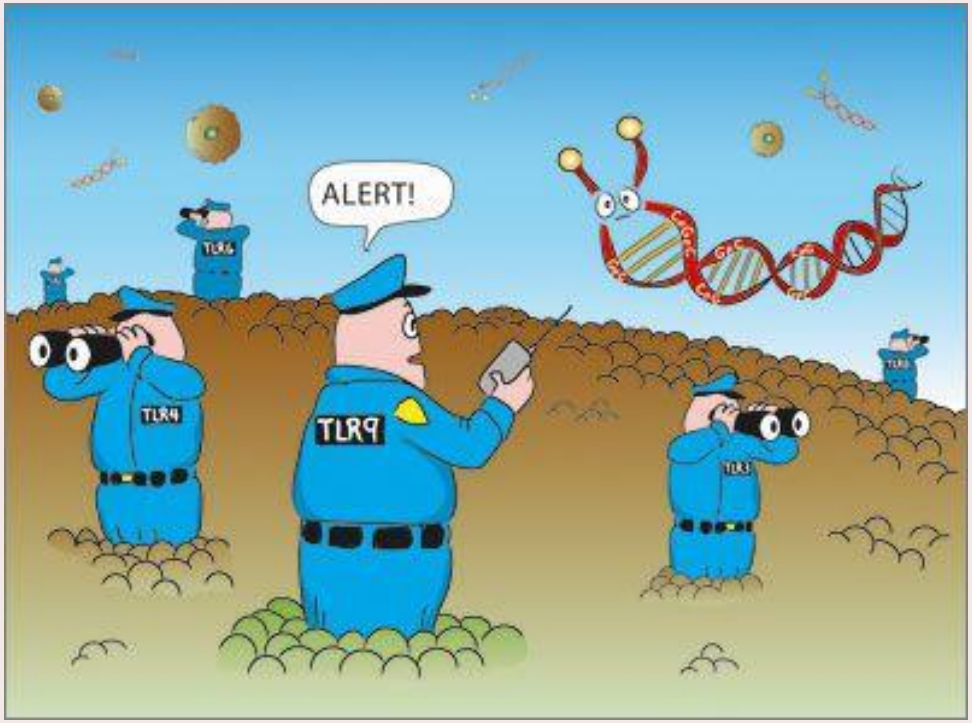
DAMP bimodal phenomenon

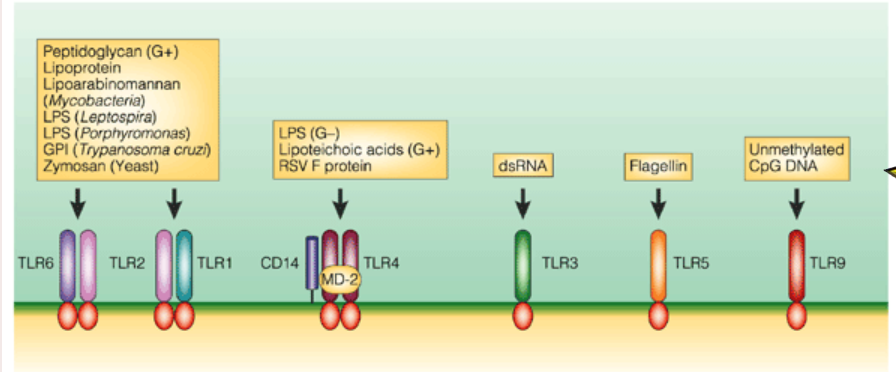
- DAMPs not only in the proinflammatory response, but also in the resolution of injury by influencing the process of tissue remodeling
- Extracellular CNA and cell-derived DNA lead to platelet activation, platelet-granulocyte interaction, and neutrophil extracellular trap formation, resulting in renal inflammation and an increase in renal injury

The criteria for establishing a candidate molecule as DAMP

- DAMP should be active as a highly purified molecule.
- Its biological activity is not owing to contamination with microbial molecules (PAMPs)
- The DAMP should be active at concentrations that are actually present in pathophysiological situations.
- Selectively eliminating or inactivating the DAMP — for example, with antibodies, specific enzymes
- Mutations or RNA interference (RNAi) — should ideally inhibit the biological activity *in vitro* and *in vivo*

What recognizes PAMP and DAMP?

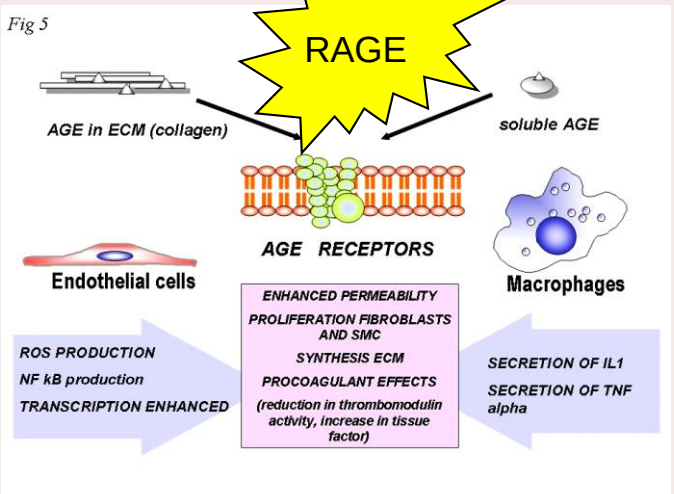




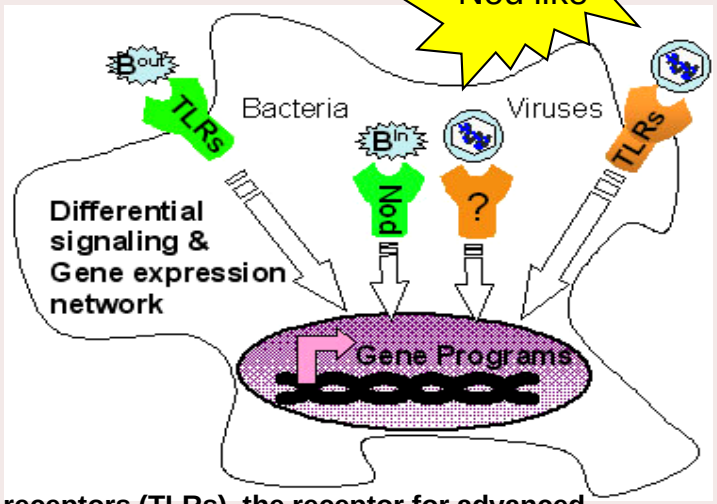
TLRs

Toll-like receptors (TLRs)

RAGE



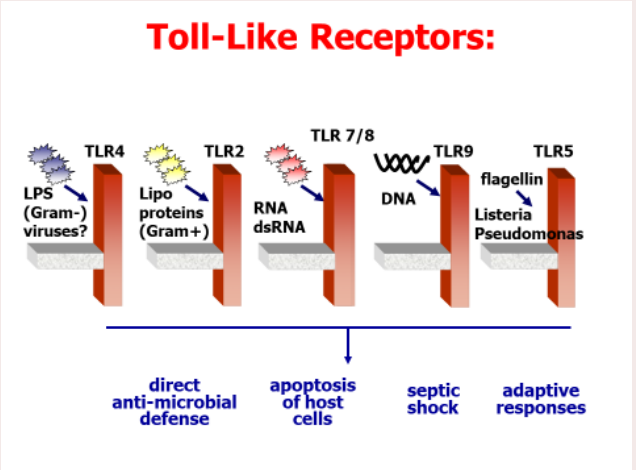
Nod like

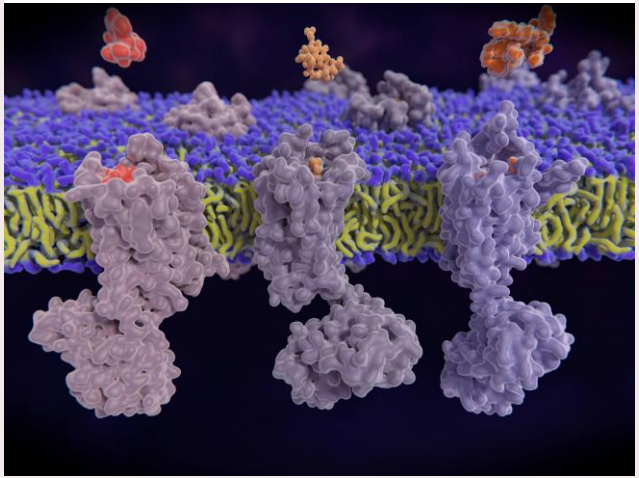


•**Danger signals mediate inflammatory responses through the Toll-like receptors (TLRs), the receptor for advanced glycation end-products (RAGE), and the NOD like receptors, after release from activated, damaged or necrotic cells.**

Toll-like receptors

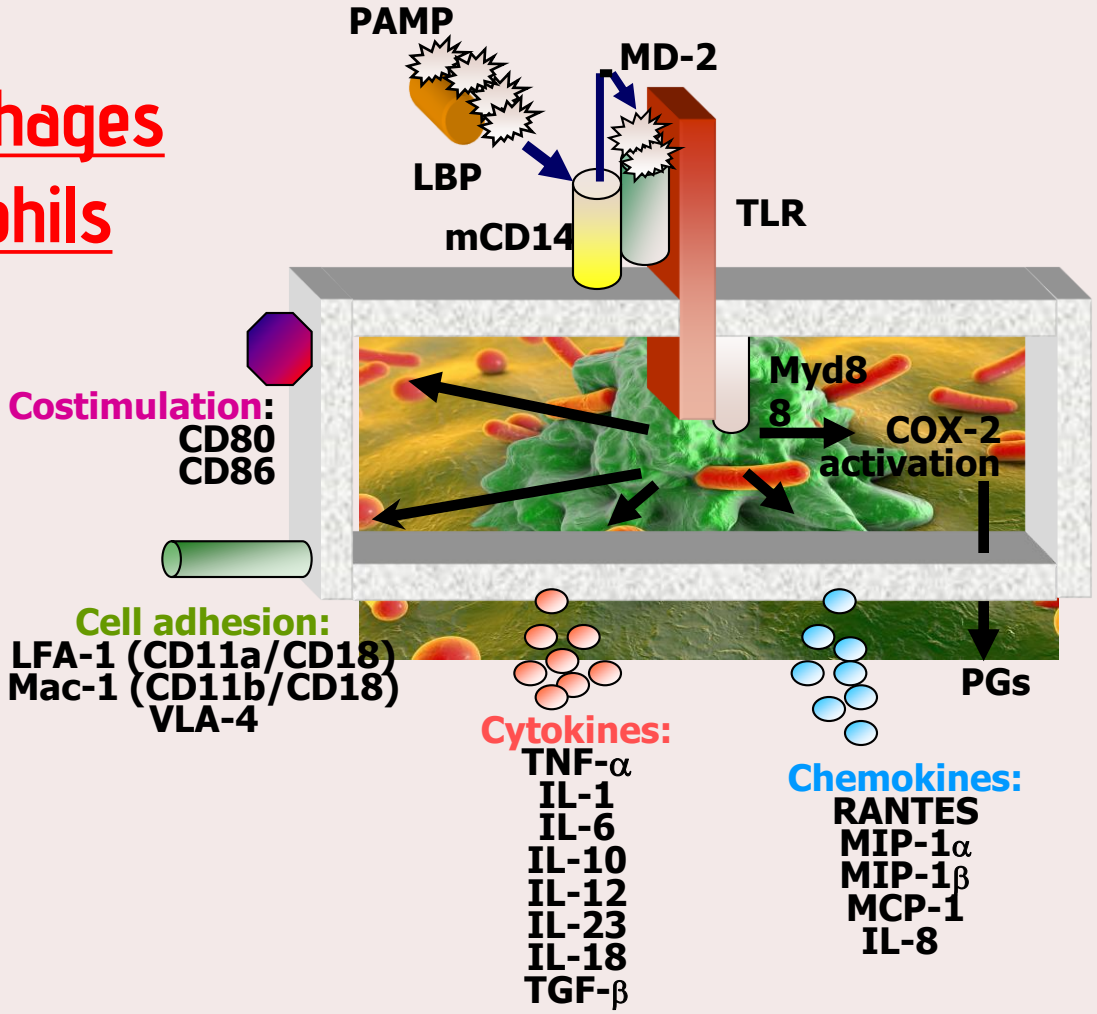
- The innate immune response is built on a class of pattern recognition receptors called Toll-like receptors (TLRs)
- The expression of TLRs are not restricted to immune tissues but distributed in all tissues including the kidney
- TLR activation leads to the release of various inflammatory cytokines
- Excessive TLR activation disrupts the immune homeostasis by sustained pro-inflammatory cytokines and chemokine production and consequently contributes to the development and progression of many diseases



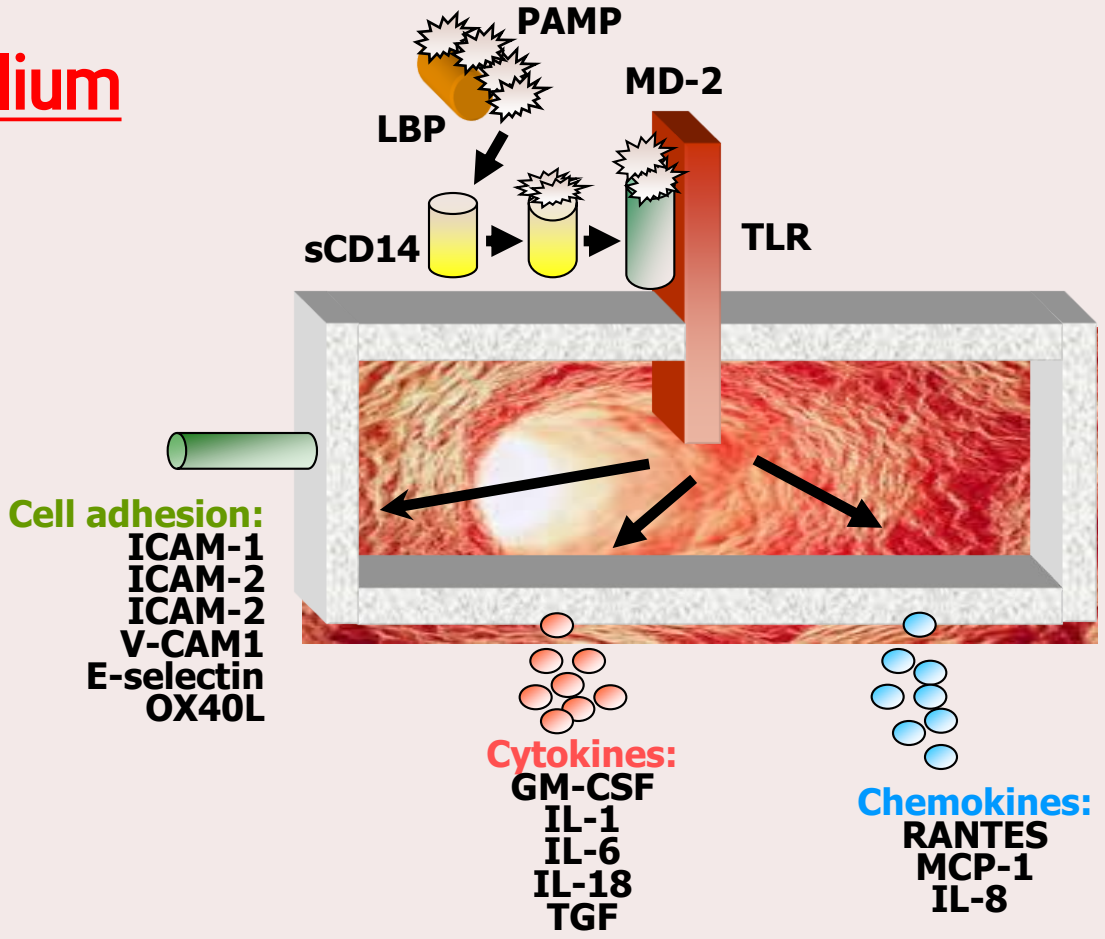


Sadik et al. 2015
Komurcu et al., 2016

Macrophages
Neutrophils

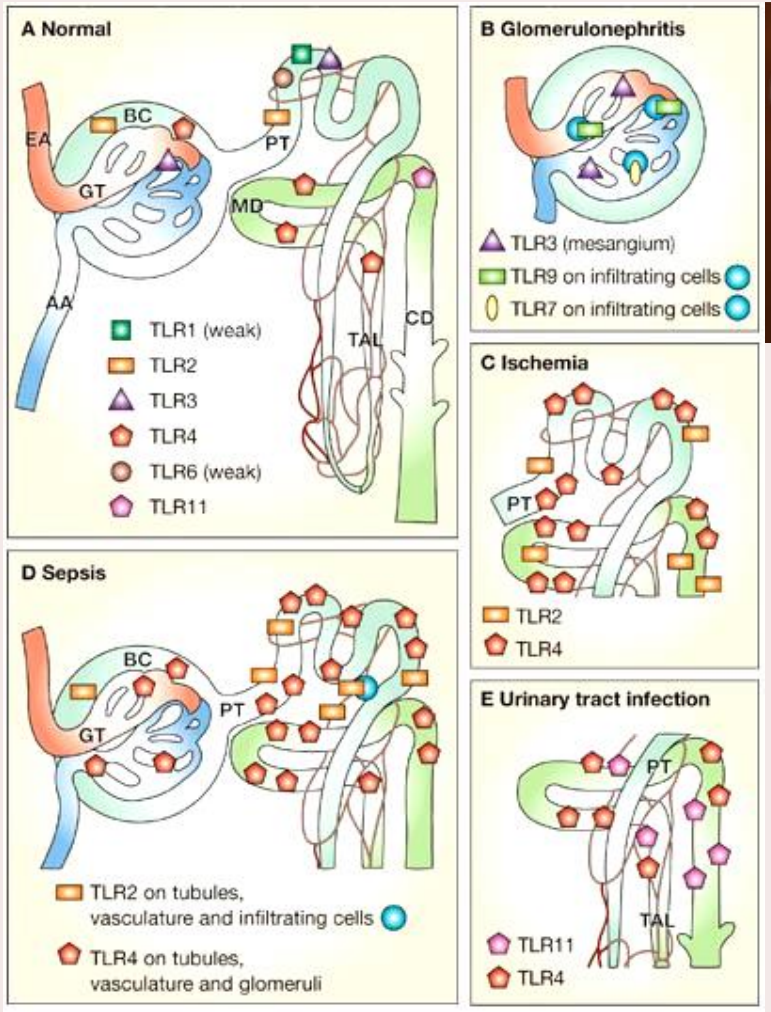


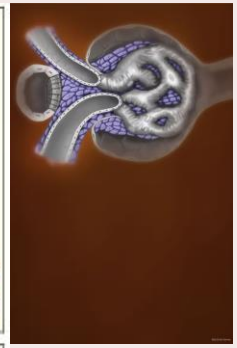
Endothelium



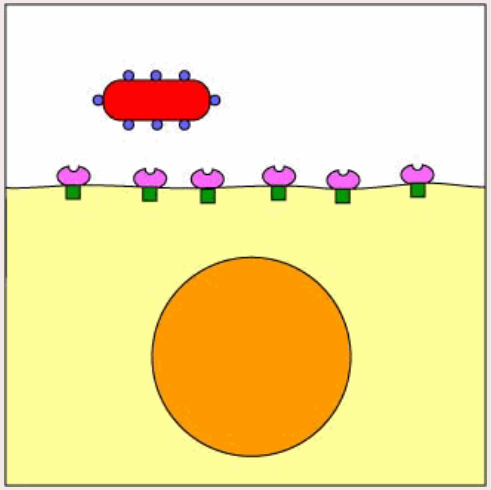
Mesangium

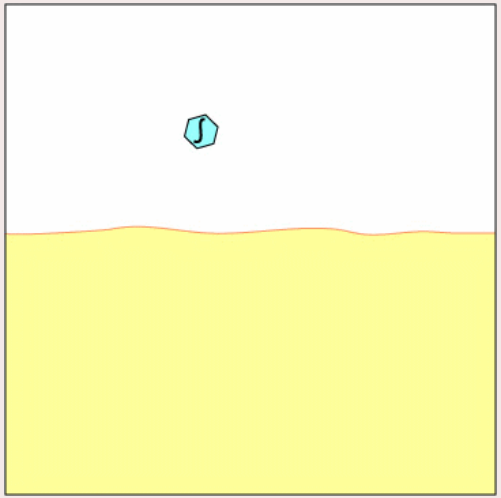
- In mesangial cells and intrarenal macrophages, at least two TLR signaling pathways can be activated capable of inducing multiple proinflammatory events, regulated by the transcription factors AP-1 and NF-kB.





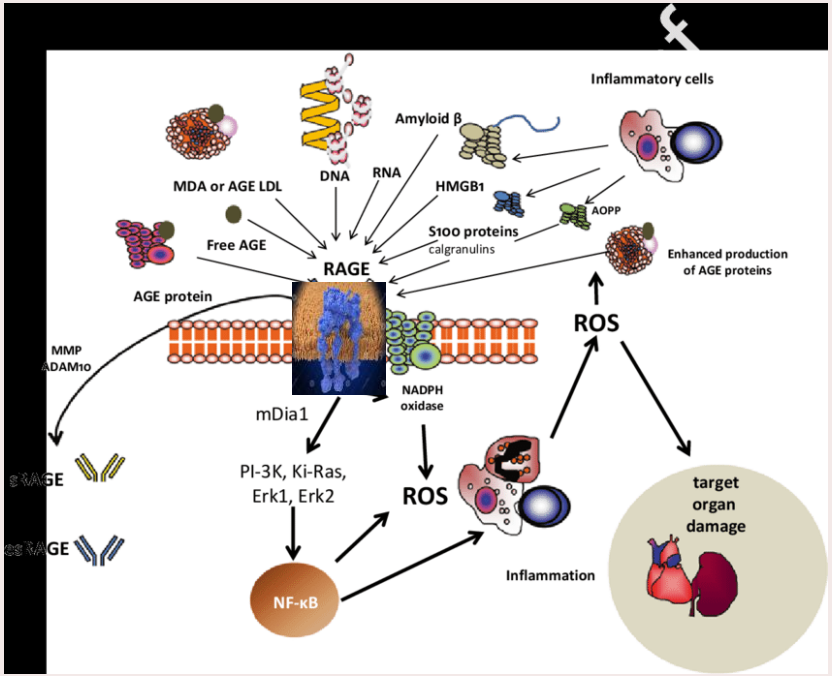
- The systemic inflammatory response initiated by PAMP exerts many similarities with the DAMP-induced systemic response.





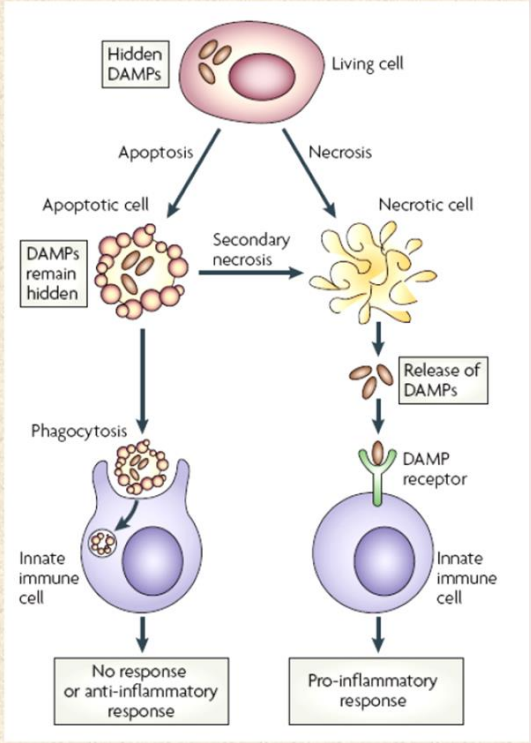
DAMP ligands of RAGE

- AGE proteins
- DNA, RNA
- HMGB1
- AOPP
- AGE-LDL
- S100 proteins
- Amyloid B
- HSPs (intracellular protein chaperones)



Renal DAMP-induced sterile inflammation

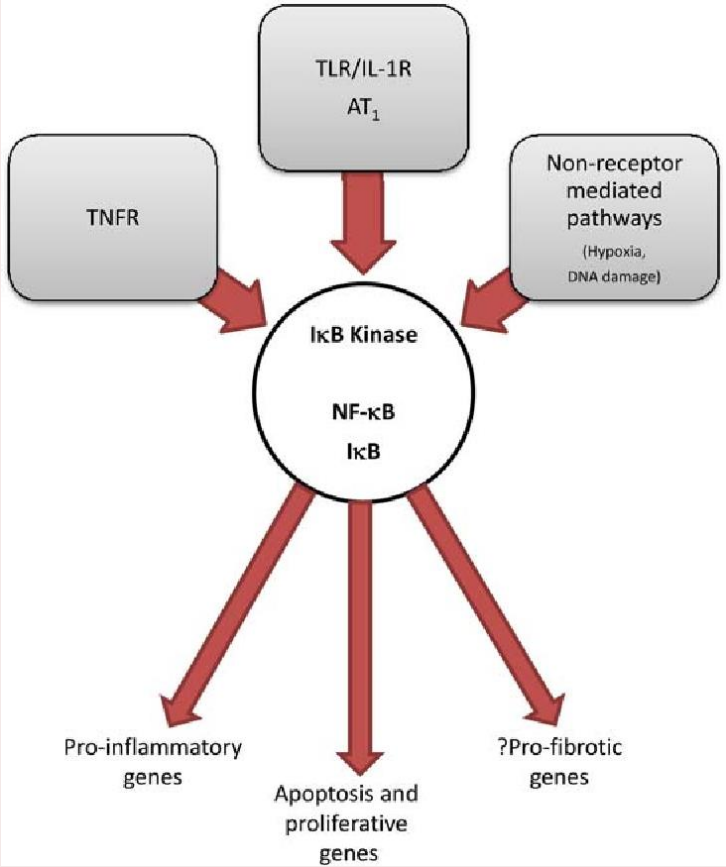
- DAMPs induce the production of chemokines, which attract neutrophils and monocytes into the tissue
- These newly recruited cells produce ROS
- They also induce microvasculature congestion, which ultimately impairs renal blood flow, creating sustained ischemia, and further contributes to increasing kidney damage
- Blockade of this very 1st step in the inflammatory cascade, thus, offers strong therapeutic potential



NF-κB in CKD

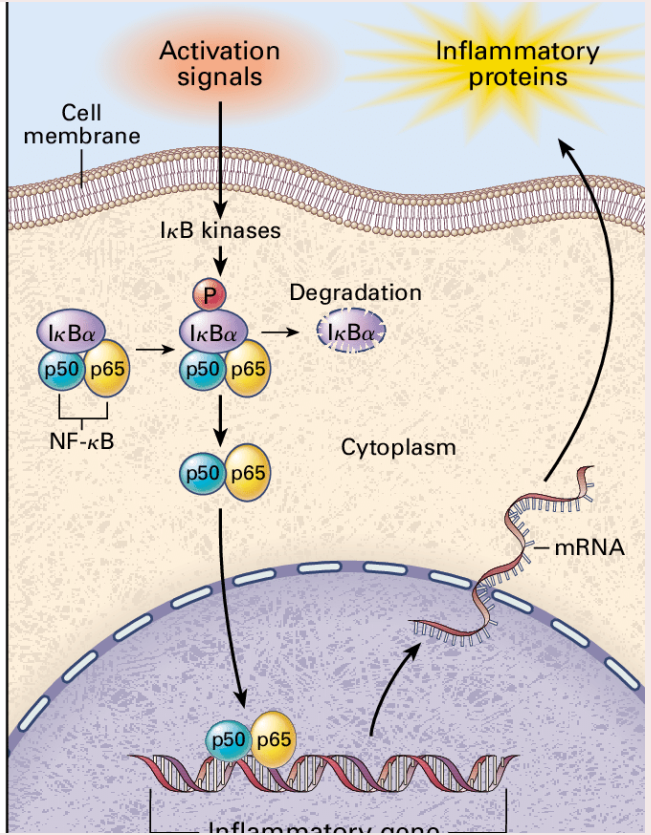
- The activation of the nuclear factor-κB (NF-κB) pathway plays a central role in the initiation and progression of inflammation, which contributes to the pathogenesis and progression of CKD

- The NF-κB family consists of five members: p50/NF-κB1, p52/NF-κB2, p65/RelA, RelB, and c-Rel, which can form a variety of homo- and hetero-dimers combinations.



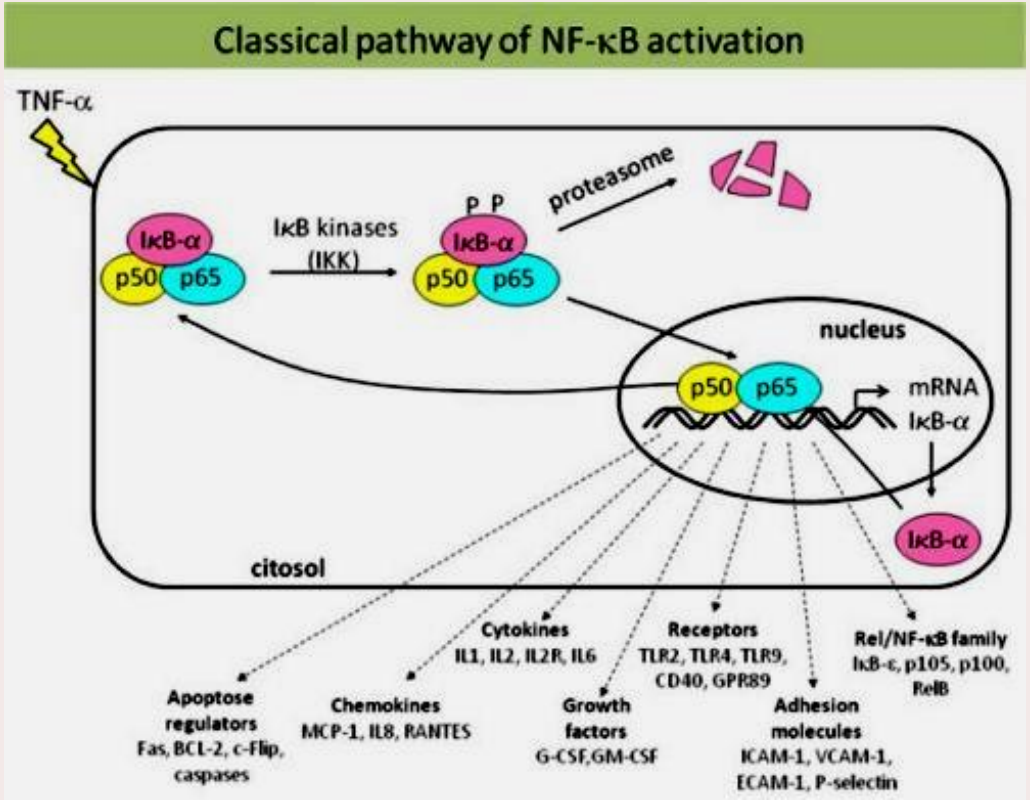
DAMP and PAMP in NF-kB activation

- Under non-inflammatory conditions, NF-κB is bound by inhibitor of kappa B (IκB) proteins in the cytoplasm.
- NF-κB is activated only under certain conditions, which include infection and injury.
- NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel are all members of the NF-κB family which allows NF-κB proteins to form dimers p50/p65 with transcriptional activity (Liu et al. 2017).



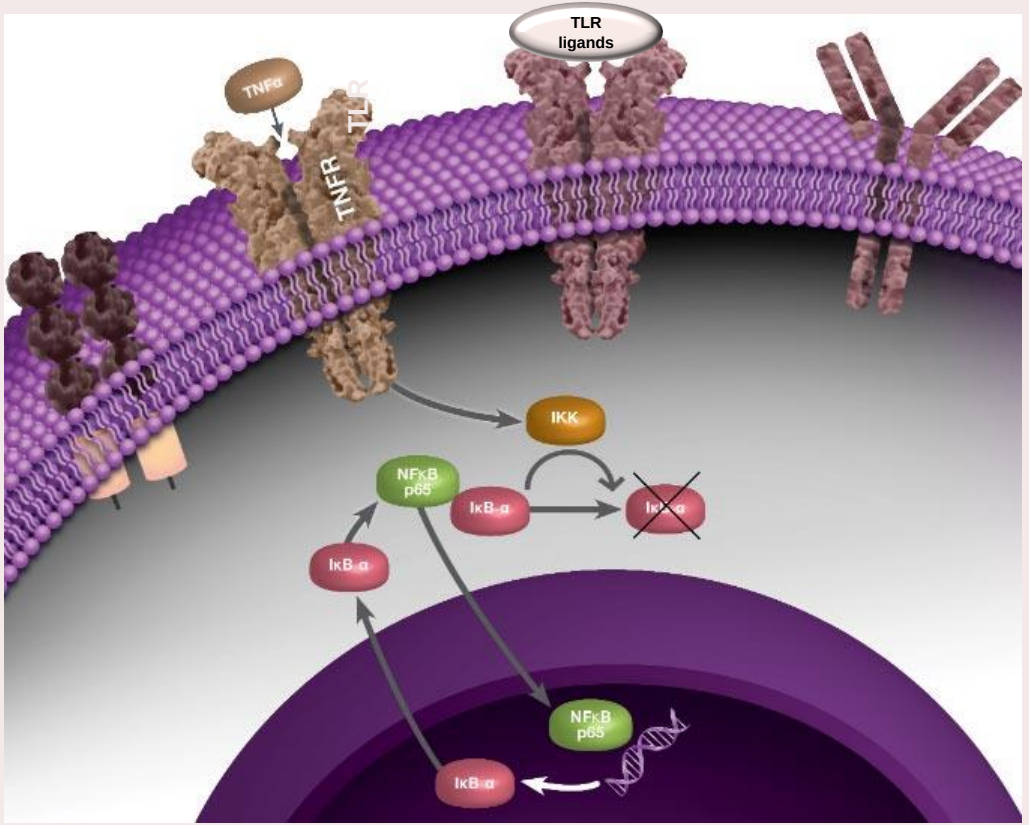
NF-kB activation

- NF-kB dimer p65 and p50 present in the cytosol linked to an inhibitory protein IkB, its degradation is through the proteasome.
- This allows NF-kB translocation into the nucleus. NF-kB is a key regulator of the inflammatory process, innate and adaptive immunity.



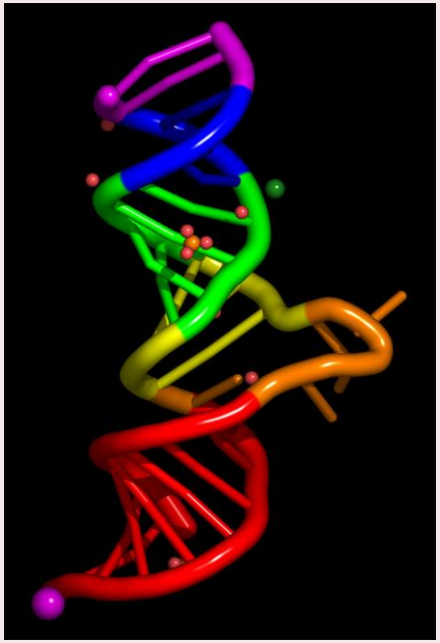
NF-kB activation

Inhibition of NF-κB signaling is often a therapeutic goal (Gupta et al. 2010).



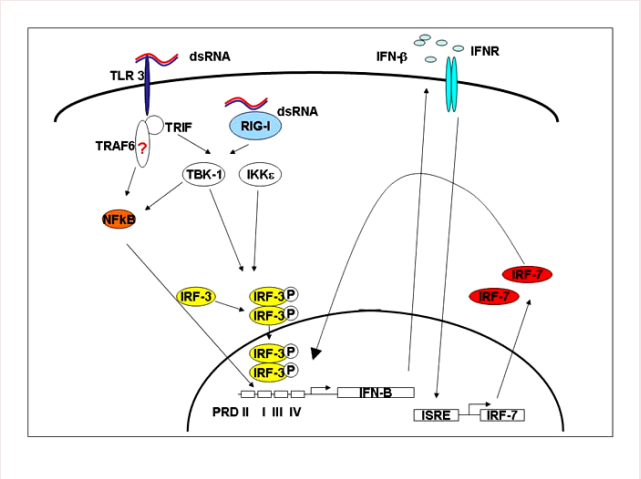
Circulating nucleic acids (CNA)

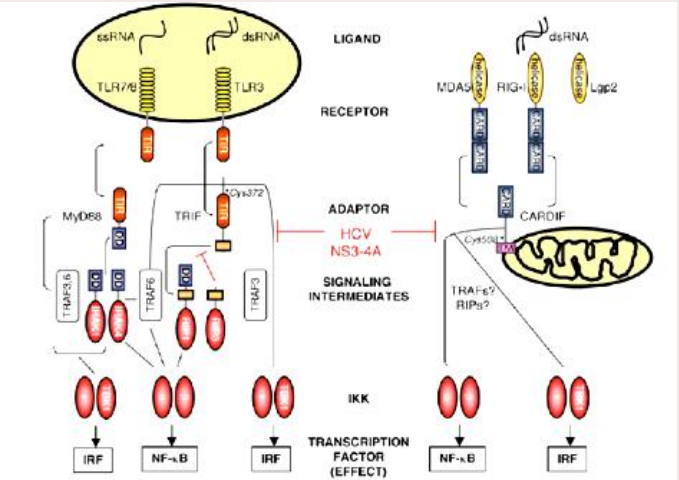
- Immunomodulatory functions recognized by a set of pattern–recognition receptors that initiate and modulate immune responses in the host
- Nucleic acids, single and double stranded, their analogs and specific “danger motifs”, represent strong TLR ligands and inducers.
- Ligands for TLR7/8 ssRNAs
- Ligands TLR3 dsRNAs
- Ligands TLR9 unmethylated CpG.



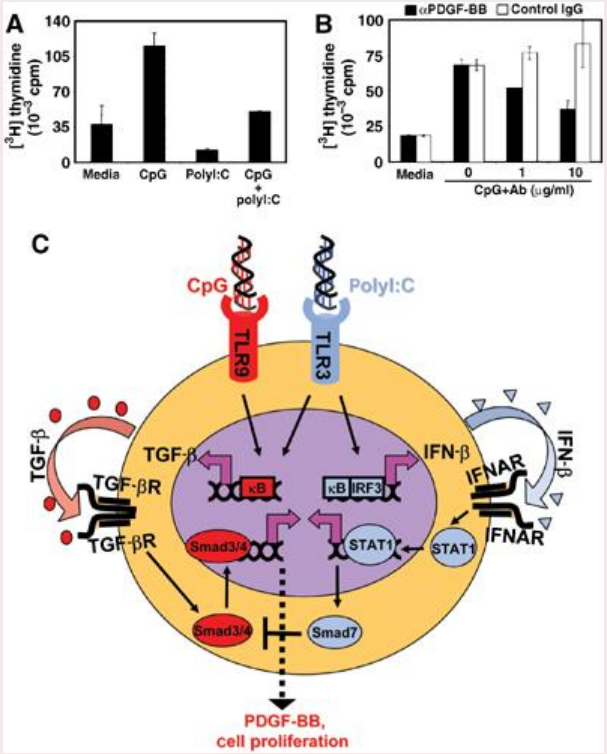
- Circulating RNA species, including different-sized dsRNAs and ssRNAs may represent critical determinants for the activation of the innate immune system, whenever they are egzogenic or endogenously generated

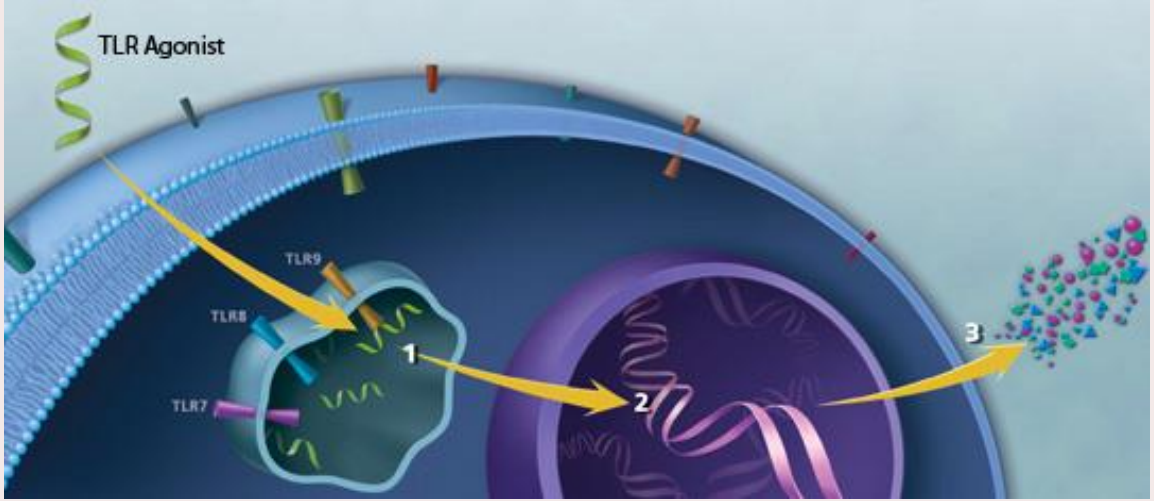
- Introduction of double stranded (ds)RNA fragments into the cytoplasm may drive normal cells to become antigen-presenting (APC)





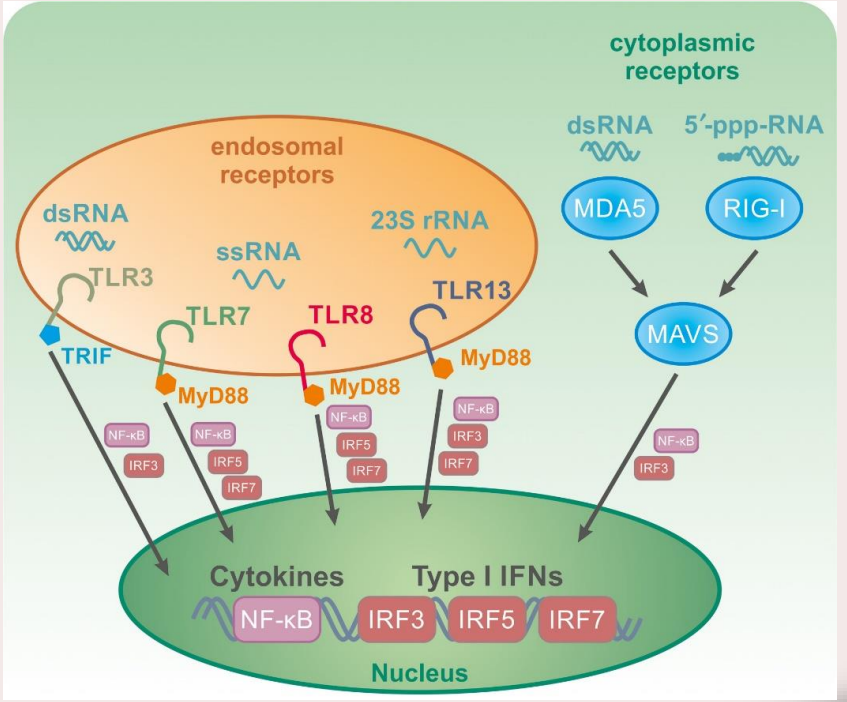
- Mesangial cells do not express TLR7/8 and TLR9.
- But injected dsRNA or CpG may be distributed to the glomerulus and then taken up by mesangial cells into intracellular endosomes in mice with experimental glomerulonephritis.
- Cultured human mesangial cells actively produce IL-6, IL-1β, IL-8, ICAM, M-CSF, and other proinflammatory factors after dsRNA uptake





- Since the TLR 3,7,8-9 are capable of recognizing diffrent-sized nucleic acids or oligonucleotides, it may allow them to sense and initiate innate and adaptive immune response in the case of nucleic acids endogenous appearance

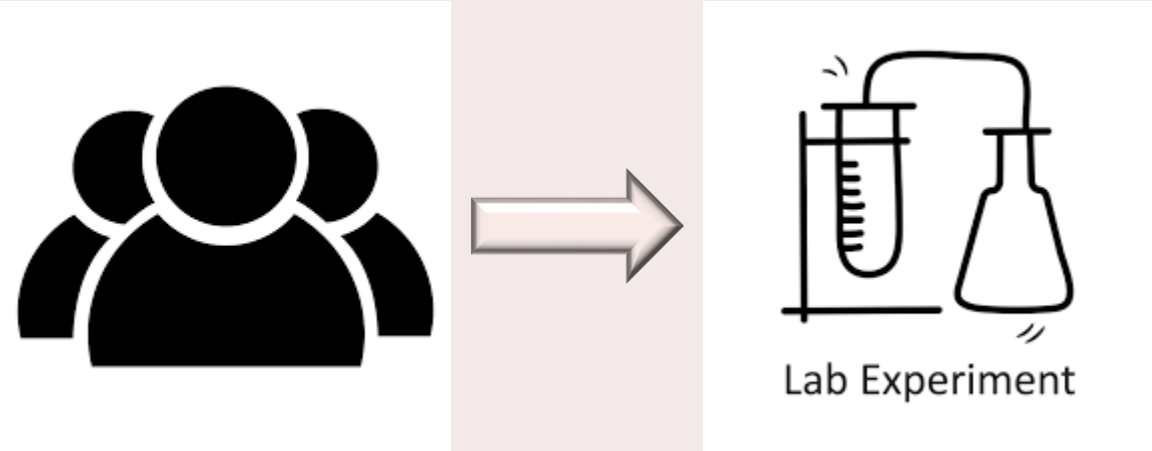
- RNA/DNA in the cytosol can activate innate immune pathways via cytosolic nucleic acid sensors.
- The ligand interaction of NA TLR activates downstream signaling cascade NF-kB, IRF3, IRF7, IRF5 and induces TNF-α, IL-6, IL-1β, IL-8, IL-12, IL-18 and chemokines
- RIG-I and MDA5 lead to enhanced IFN response



- A family of extracellular RNases and DNases are involved in degradation of all CNA or oligonucleotide derivatives.
- In the presence of extracellular nucleases, CNA can hardly reach APC

Our study

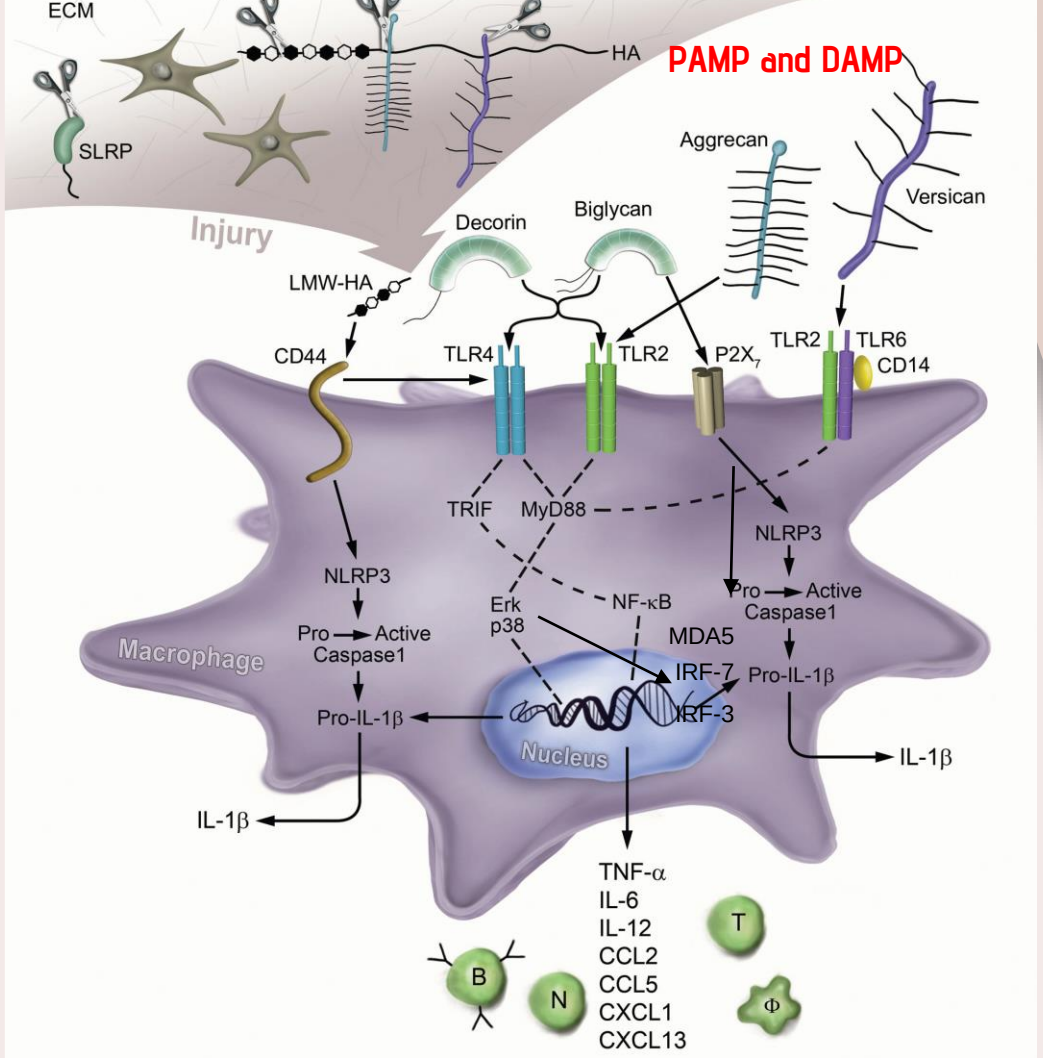
We hypothesized that the available CNA, acting as DAMPs, may be a possible pathogenetic mechanism capable of modulating immune-inflammatory reaction in patients with CRF



Determination:

NF-κBp65 (C-20)
P38
MDA-5
IRF-3
IRF-7

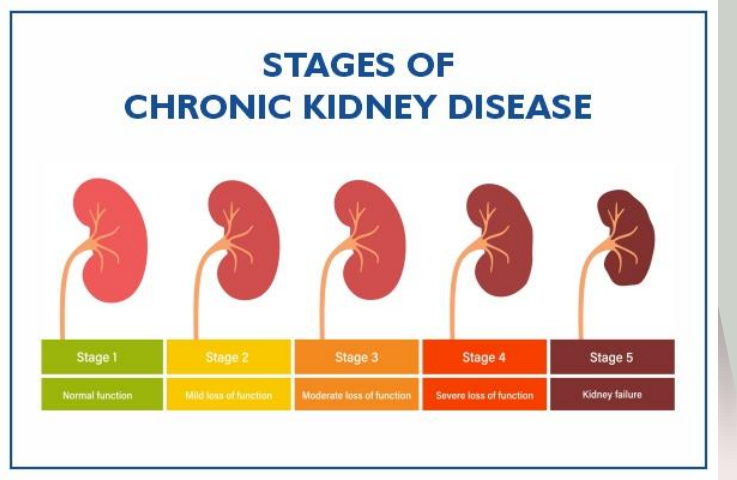
purchased from Santa Cruz Biotechnology (CA, USA)



Patients and control subjects:

- Patients with the different stages of chronic kidney disease were selected according to the National Kidney Foundation (NKF) criteria, recruited from the Clinic for Nephrology and hemodialysis Medical Faculty, University of Nis:

- Stage 1: Kidney damage with normal or increased GFR
- Stage 2: Mild reduction in GFR
- Stage 3: Moderate reduction in GFR
- Stage 4: Severe reduction in GFR
- Stage 5: Kidney failure, patient on dialysis program
- Transplanted patients



Isolation of nucleic acids and oligonucleotide samples

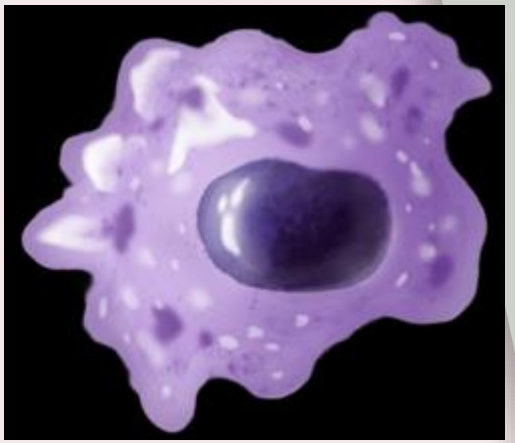
- For the isolation of RNA from plasma samples, the commercial RNA isolation reagent set TRI Reagent BD (T3809) for the simultaneous isolation of RNA, DNA and proteins from plasma was used, purchased from Sigma (Saint Louis, USA).

- The concentration of circulating nucleic acids was calculated by using corresponding standard of RNA. The UV spectra obtained in our study are in a close agreement with recent revised UV extinction coefficients of RNA and related nucleotides

Culture of residental macrophages

The passaged residential macrophages were obtained from the Department of Microbiology, Biochemistry, and Biotechnology, University of Maribor (Slovenia). PBMCs were isolated from buffy coats of healthy blood donors after Ficoll centrifugation, allowed to differentiate into monocyte-derived macrophages.

DMEM, supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, 10% heat-inactivated FCS , and 1 mM glutamine, was used for cultivation. Residential macrophages, obtained after 64th passage, cultured in 24-well plates, allocated into different groups:



I the cells exposed to media supplemented with 100 µL of physiological saline solution;
II the cells exposed to media supplemented with 100 µL of RNAs purified from plasma of control healthy subjects;
III the cells exposed to media supplemented with 100 µL of RNAs purified from different groups of patients (1-5 stages of CRF, dyalysis and tarnsplanted pat)
The number of samples of each group corresponded to the number of circulating nucleic acid samples, except that untreated group of cells exposed to media and physiological saline solution consisted of 10 samples.

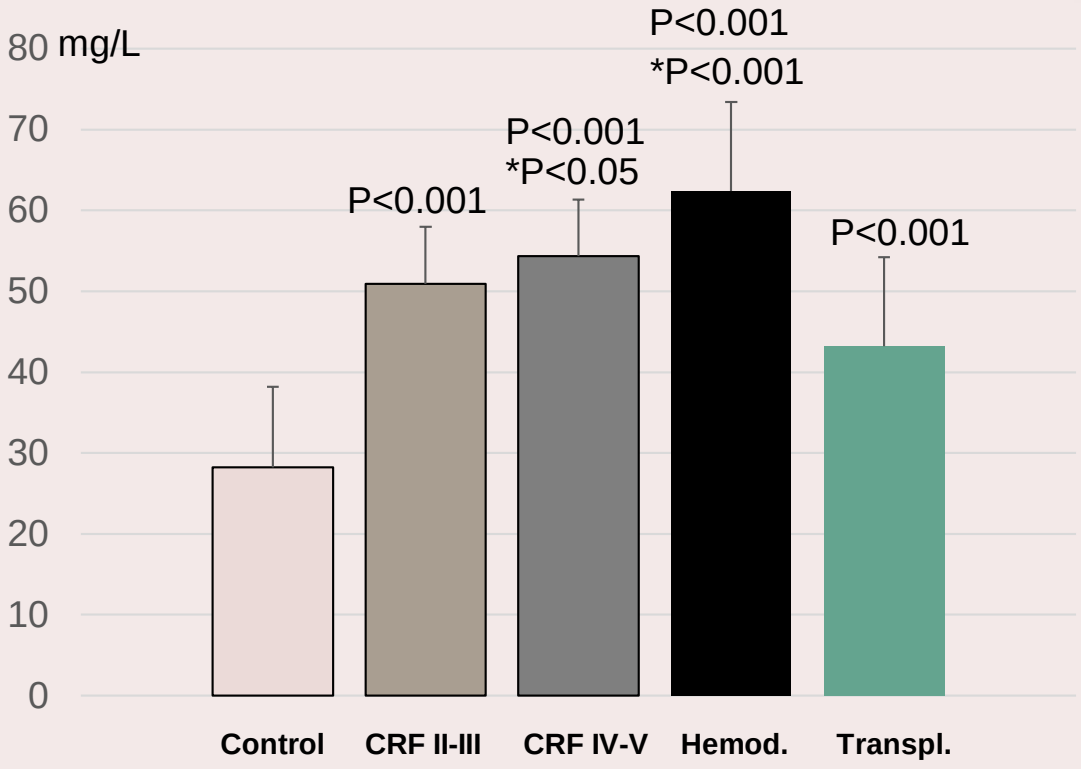
Stage/type of renal disease	N	Urea (mmol/L)	Creatinine (μmol/L)	Circulating RNA (mg/L)
Control	14	3.56 ± 1.39	76.96 ± 8.22	28.2 ± 10.33
Chronic renal failure (stage II–III)	10	24.35 ± 10.70	315.68 ± 45.56	50.95 ± 10.11
		$p < 0.001$	$p < 0.001$	$p < 0.001$
		$p < 0.001$**	$p < 0.001$**	
Chronic renal failure (stage IV–V)	9	34.2 ± 8.36	572 ± 107.51	54.37 ± 7.25
		$p < 0.001$	$p < 0.001$	$p < 0.001$
		$p < 0.001$**	$p < 0.001$**	$p < 0.05$**
Renal hemodialysis	13	21.83 + 8.05	664.43 + 151.09	62.4 ± 12.78
		$p < 0.001$	$p < 0.001$	$p < 0.001$
		$p < 0.001$**	$p < 0.001$**	$p < 0.001$**
Renal transplantation	6	13.13 ± 5.20	186.96 ± 60.22	43.25 ± 11.23
		$p < 0.001$	$p < 0.001$	$p < 0.001$

Notes: Patients with the different stages of chronic kidney disease were recruited from the Clinic for Nephrology and Hemodialysis Medical Faculty, University of Nis. Blood laboratory markers of kidney damage were present in establishing a diagnosis of stages of chronic kidney disease. They were selected according to the National Kidney Foundation (NKF) criteria.

*$p < 0.001$, statistical significance compared to the control.

**$p < 0.001$, statistical significance compared to the patients who underwent renal transplantation.

Kocić G et al. Circulating nucleic acids as possible damage-associated molecular patterns in different stages of renal failure. Ren Fail. 2010;32(4):486-92.



Circulating nucleic acids concentration in groups of patients

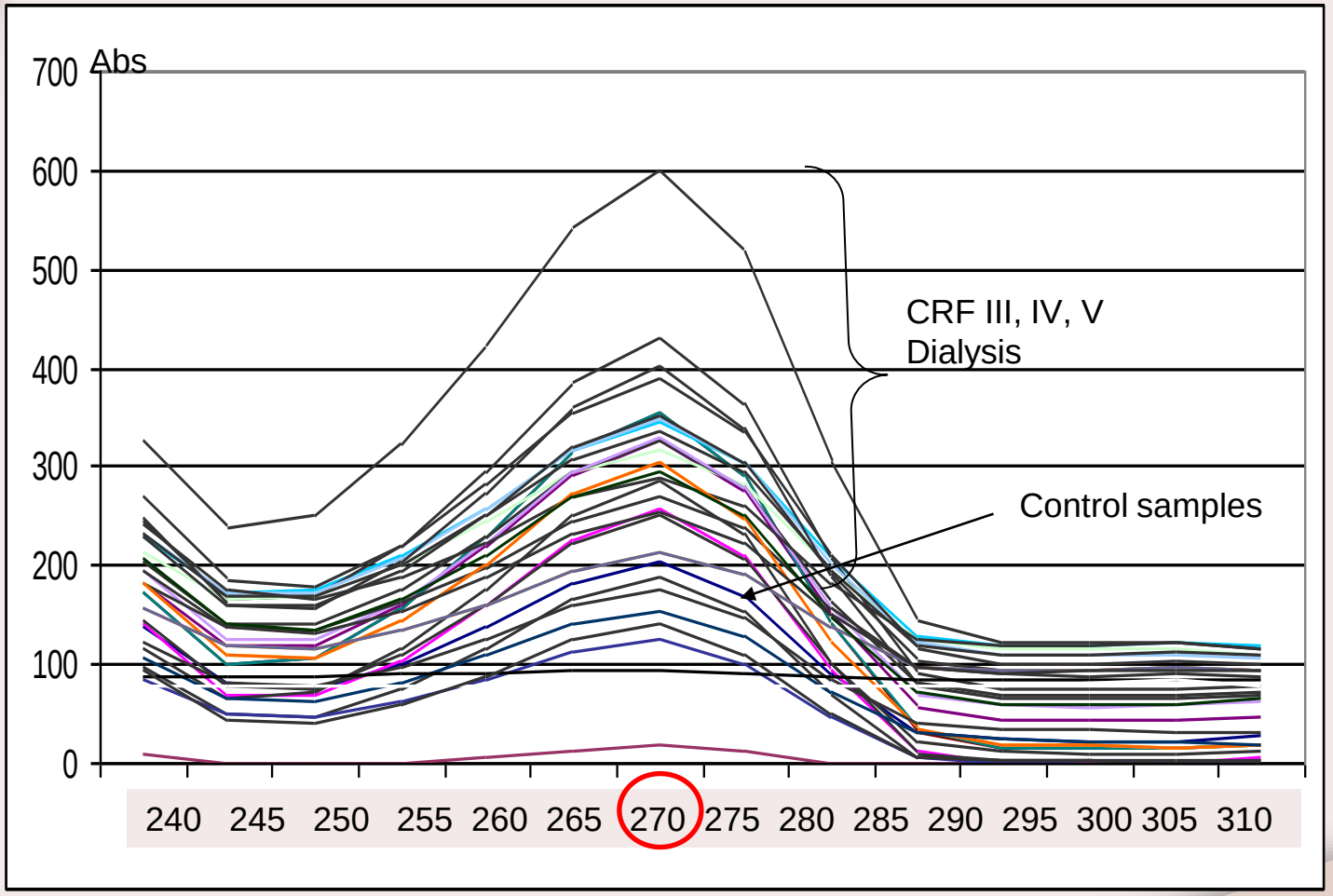


Figure 2. Spectrophotometric scan analysis of plasma nucleic acids

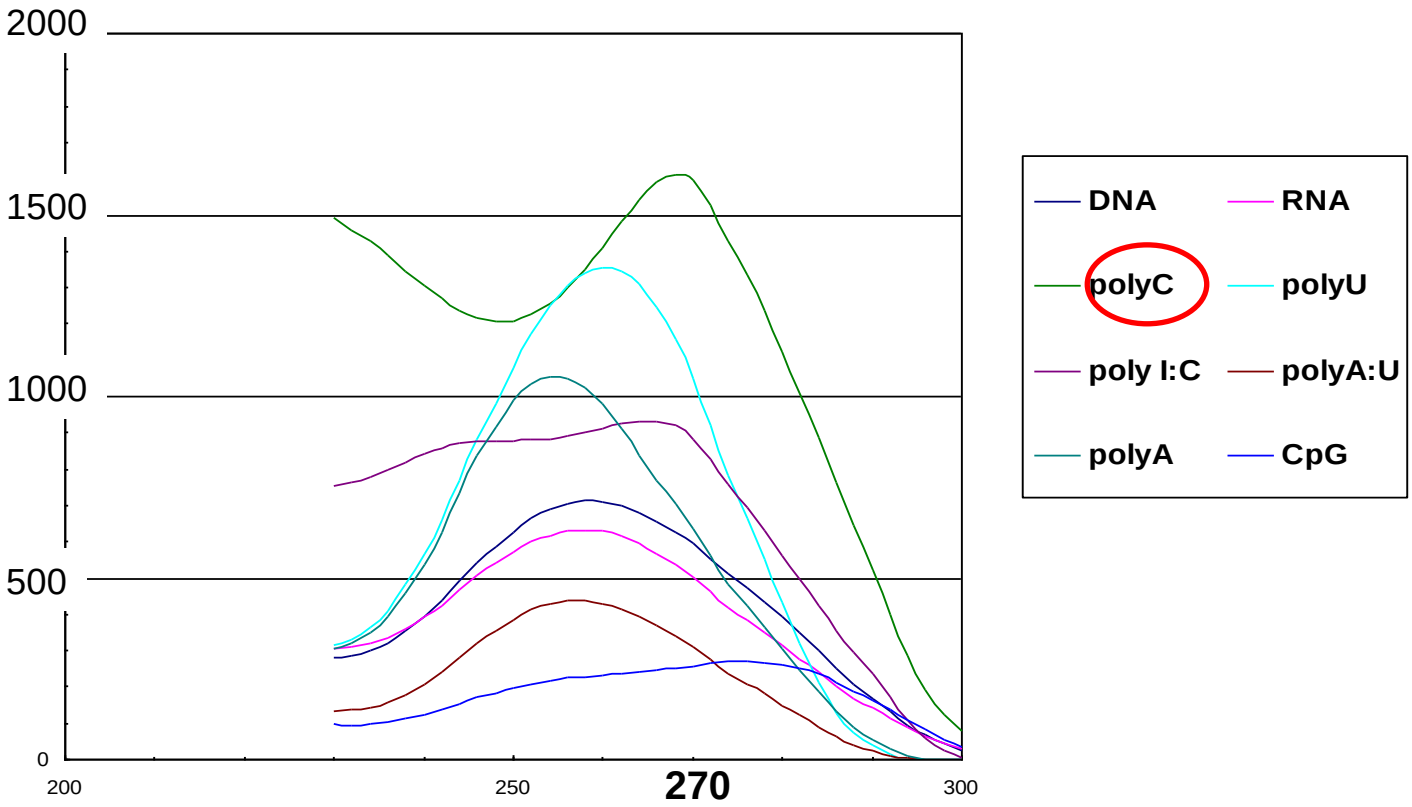
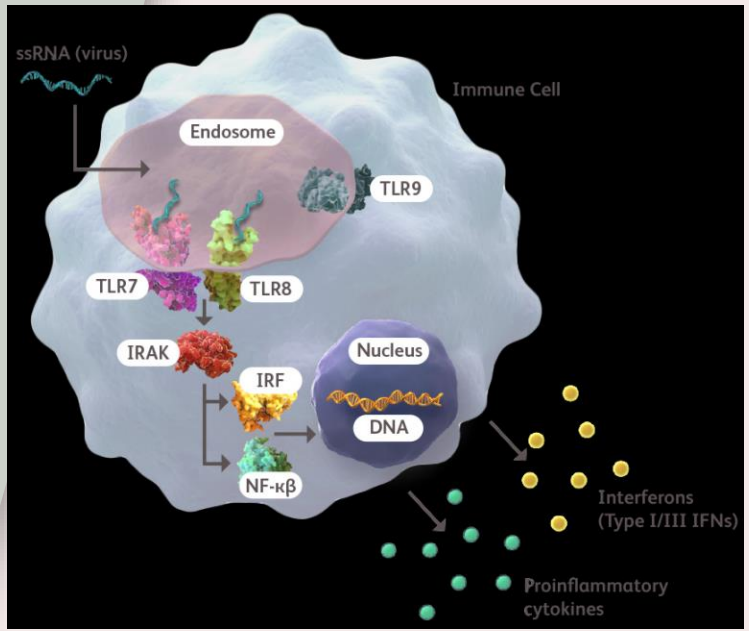
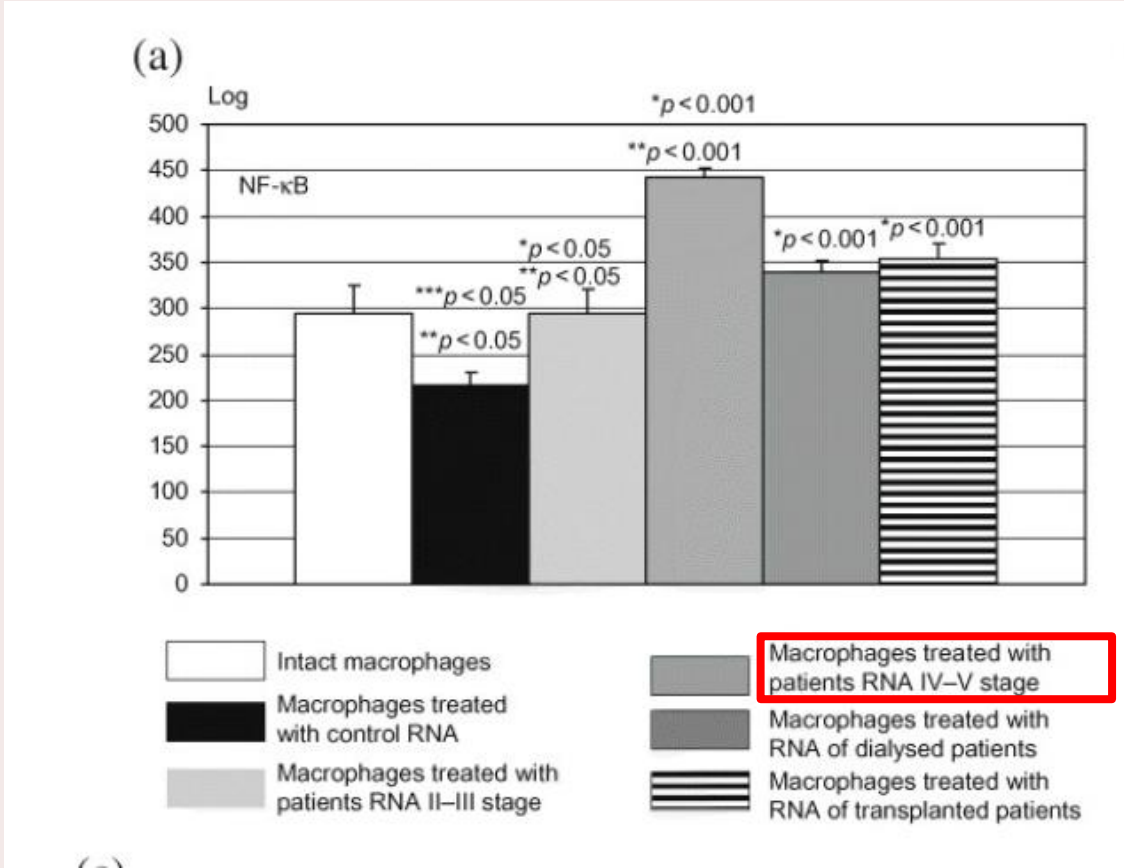
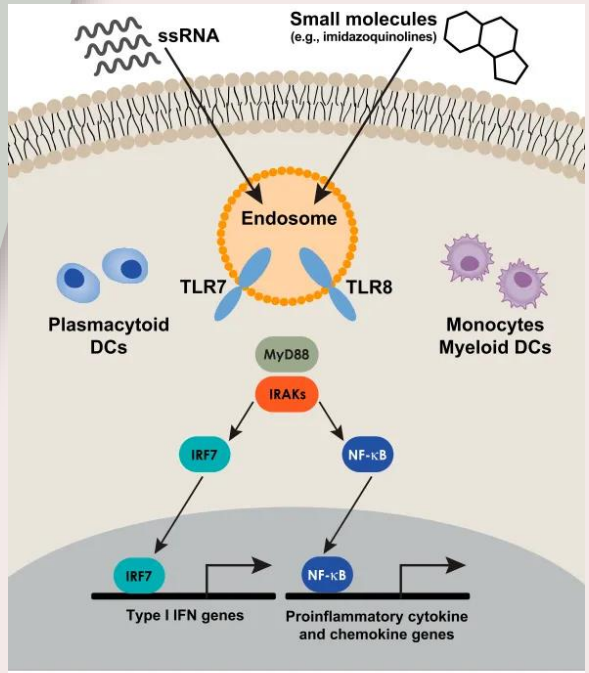


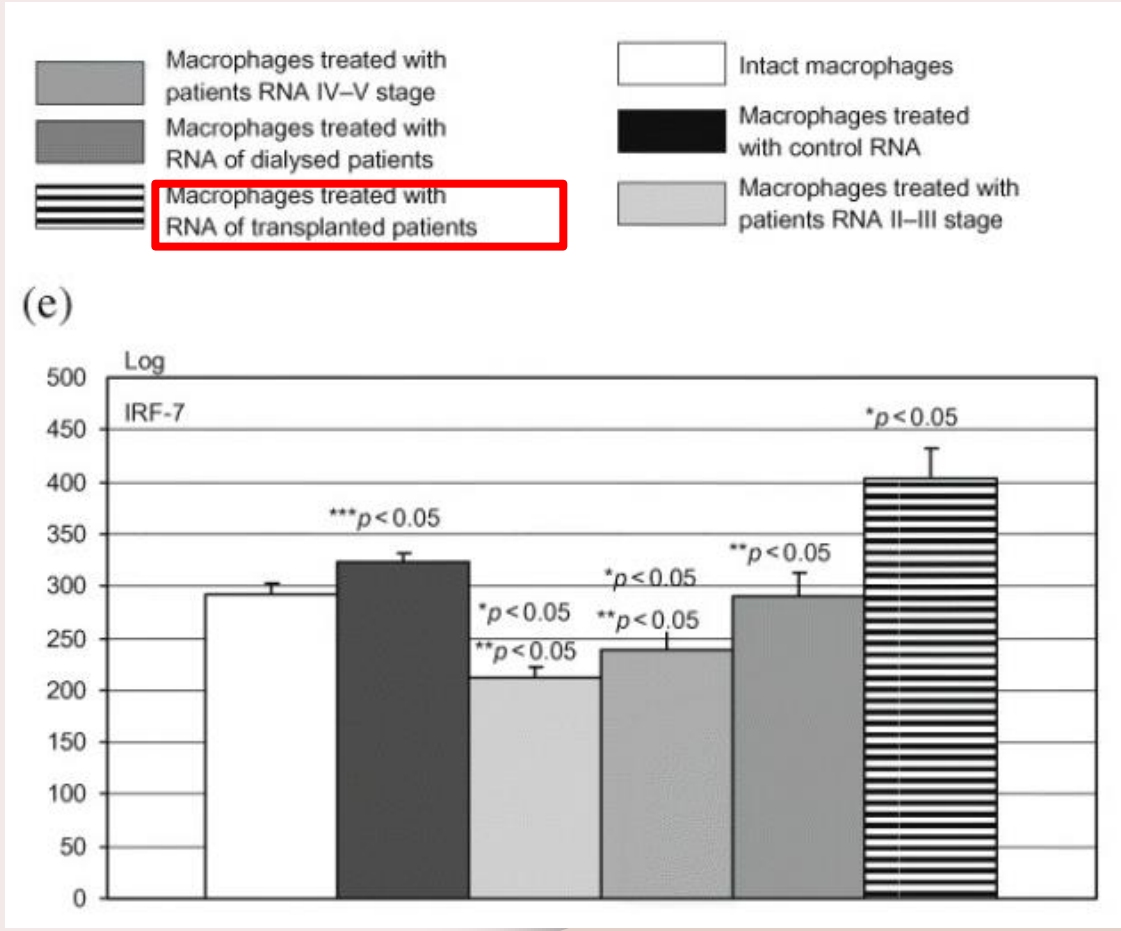
Figure 3. Spectrophotometric scan analysis of corresponding standards


ssRNA (virus)
Immune Cell
Endosome
TLR9
TLR7
TLR8
IRAK
IRF
Nucleus
DNA
NF-κβ
Interferons
(Type I/III IFNs)
Proinflammatory
cytokines

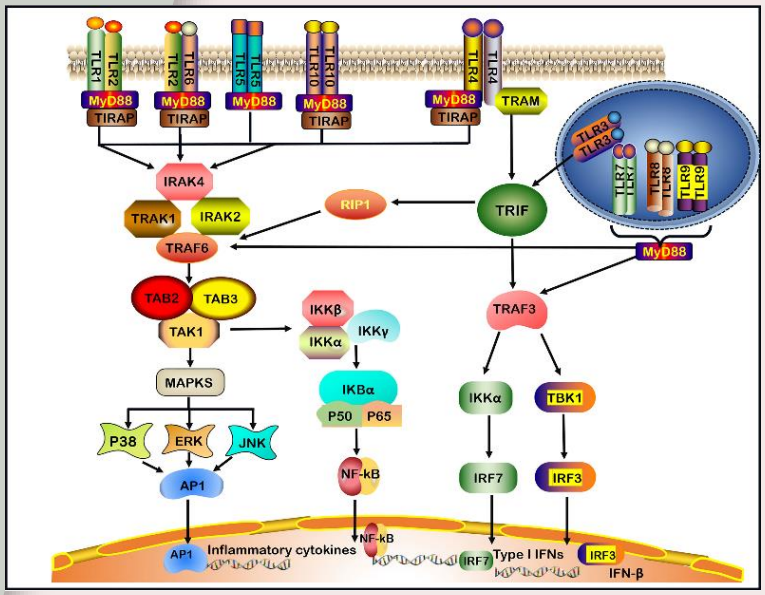

(a)
Log
NF-κB
500
450
400
350
300
250
200
150
100
50
0
*p < 0.001
**p < 0.001
*p < 0.001
*p < 0.001
*p < 0.05
**p < 0.05
***p < 0.05
**p < 0.05
Intact macrophages
Macrophages treated with control RNA
Macrophages treated with patients RNA II–III stage
Macrophages treated with patients RNA IV–V stage
Macrophages treated with RNA of dialysed patients
Macrophages treated with RNA of transplanted patients

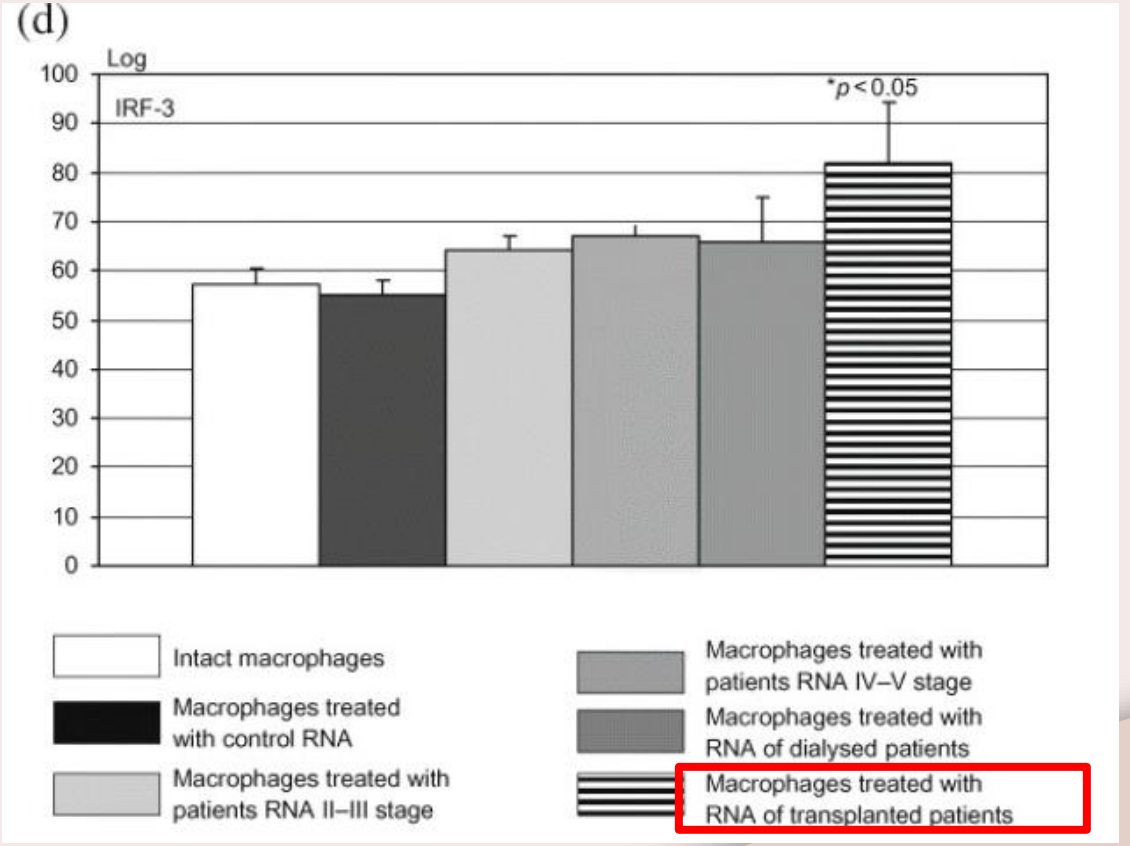


D. J. Dowling, "Recent Advances in the Discovery and Delivery of TLR7/8 Agonists as Vaccine Adjuvants," ImmunoHorizons, vol. 2, no. 6, pp. 185–197, 2018, doi: 10.4049/immunohorizons.1700063.

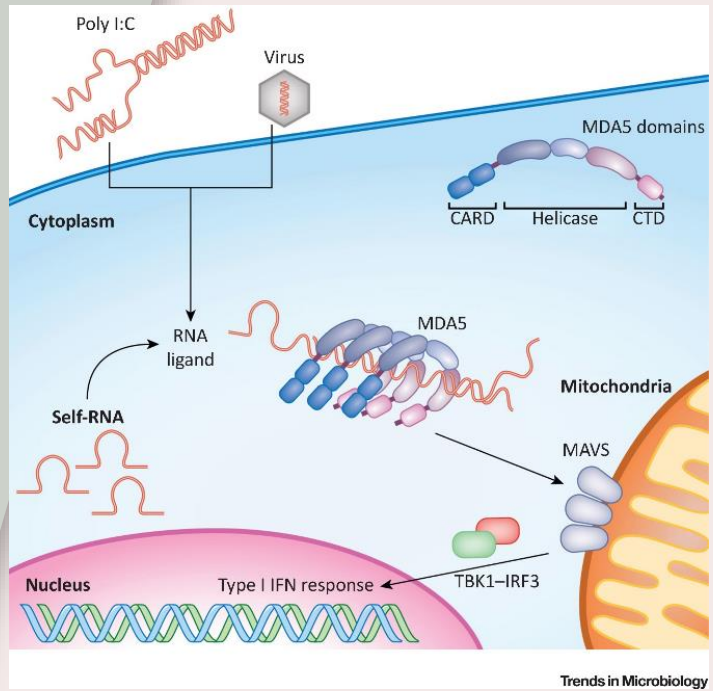


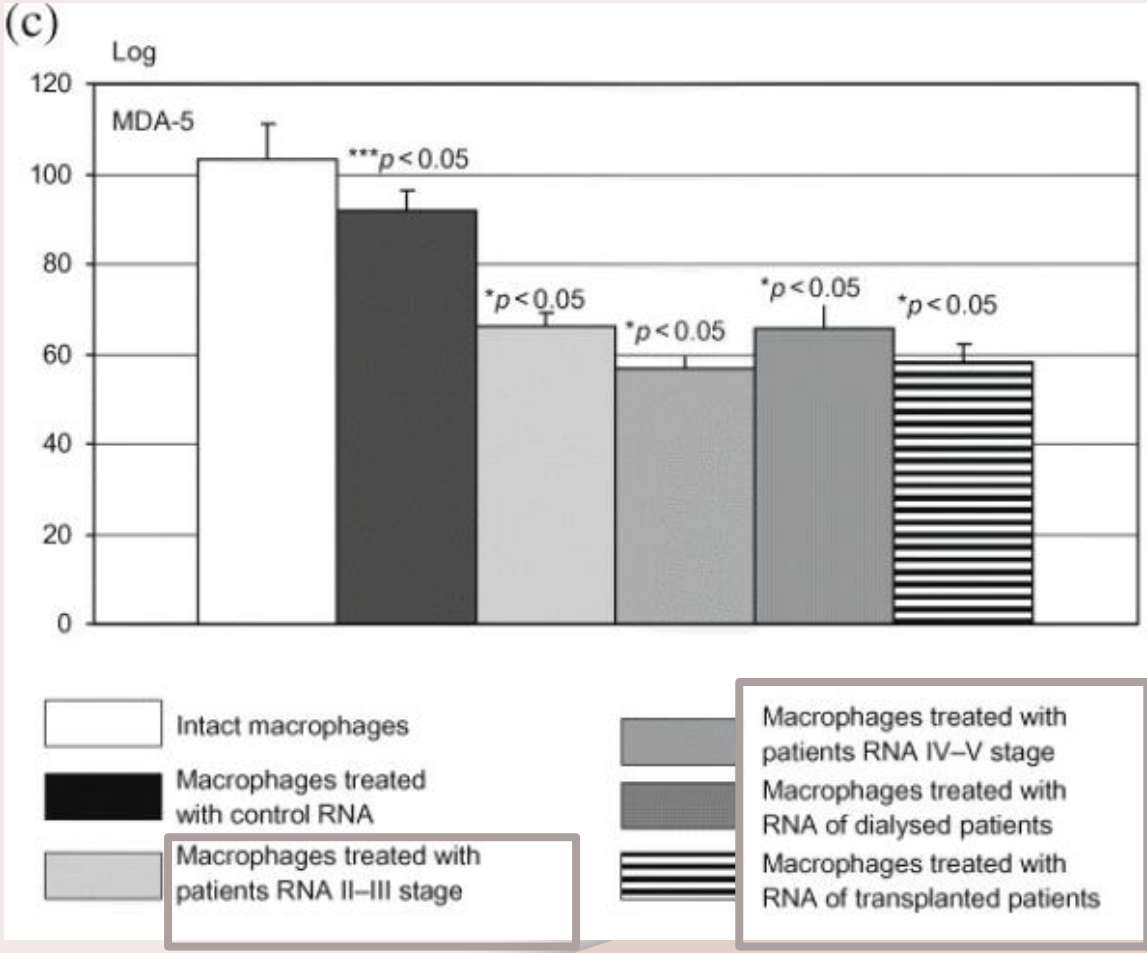
Use of interferon in kidney transplant is not advised, since it increases the chance of episodes of acute humoral rejection (15-64%) three to six months after beginning treatment (Fabrizi et al Aliment Pharmacol Ther 2006;24(10):1413-22)

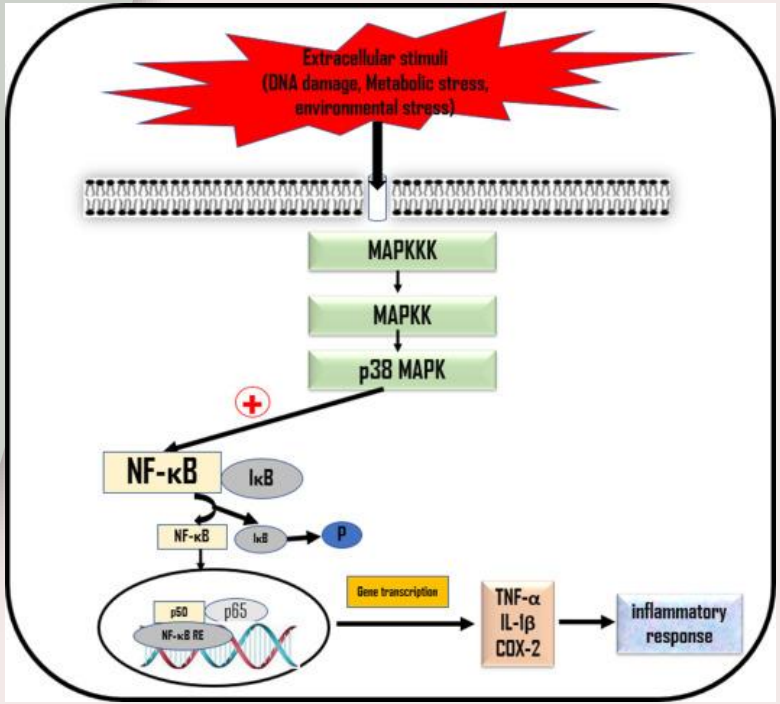


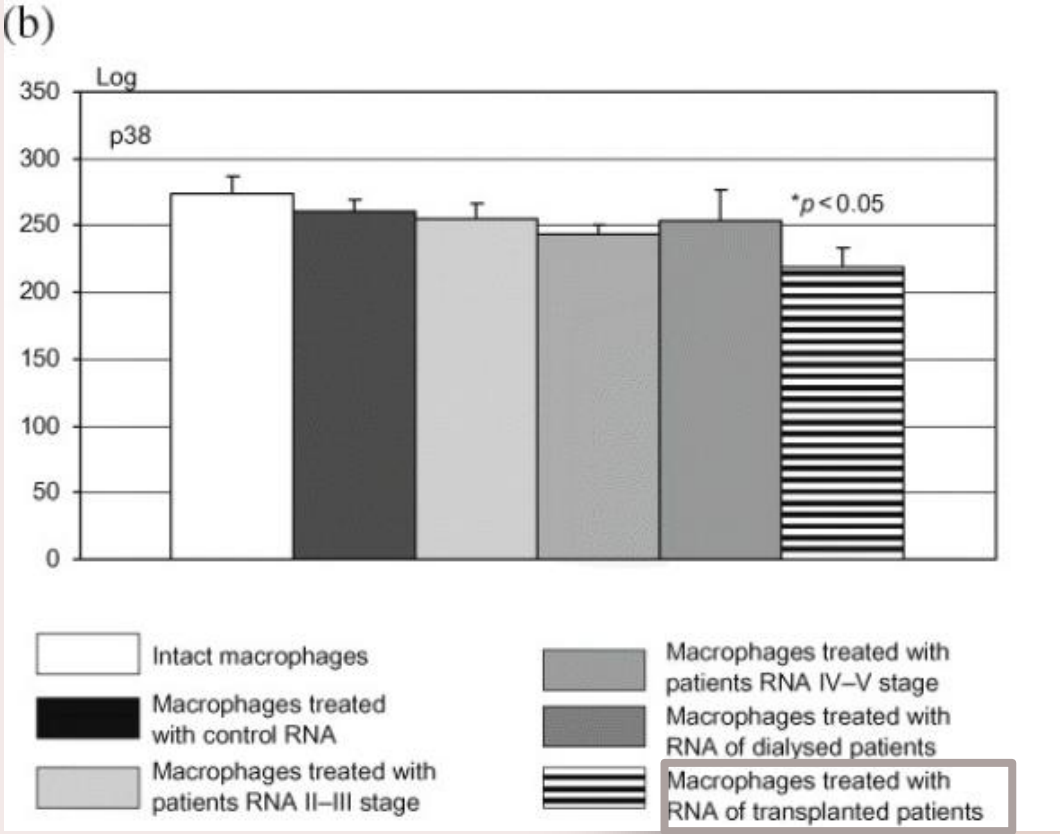


Lichtnekert J etal. Trif is not required for immune complex glomerulonephritis: dying cells activate mesangial cells via Tlr2/Myd88 rather than Tlr3/Trif. Am J Physiol Renal Physiol 2009; 296: F867–F874.


Poly I:C
Virus
MDA5 domains
CARD
Helicase
CTD
Cytoplasm
RNA ligand
MDA5
Mitochondria
Self-RNA
MAVS
Nucleus
Type I IFN response
TBK1–IRF3
Trends in Microbiology


(c)
Log
MDA-5
120
100
80
60
40
20
0
***p < 0.05
*p < 0.05
*p < 0.05
*p < 0.05
*p < 0.05
Intact macrophages
Macrophages treated with control RNA
Macrophages treated with patients RNA II–III stage
Macrophages treated with patients RNA IV–V stage
Macrophages treated with RNA of dialysed patients
Macrophages treated with RNA of transplanted patients


Extracellular stimuli
(DNA damage, Metabolic stress,
environmental stress)
MAPKKK
MAPKK
p38 MAPK
NF-κB
IκB
NF-κB
IκB
P
p50
p65
NF-κB RE
Gene transcription
TNF-α
IL-1β
COX-2
inflammatory
response

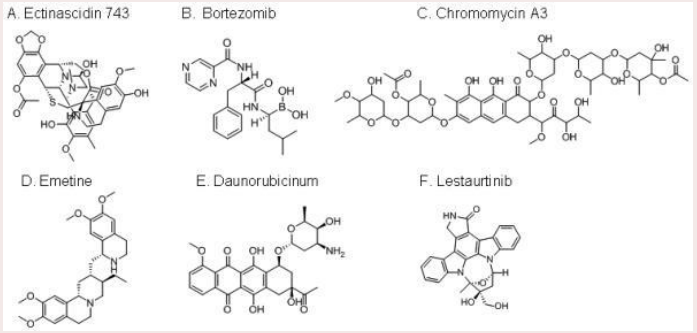

(b)
Log
p38
350
300
250
200
150
100
50
0
*p < 0.05
Intact macrophages
Macrophages treated with control RNA
Macrophages treated with patients RNA II–III stage
Macrophages treated with patients RNA IV–V stage
Macrophages treated with RNA of dialysed patients
Macrophages treated with RNA of transplanted patients

NFκB in CRF

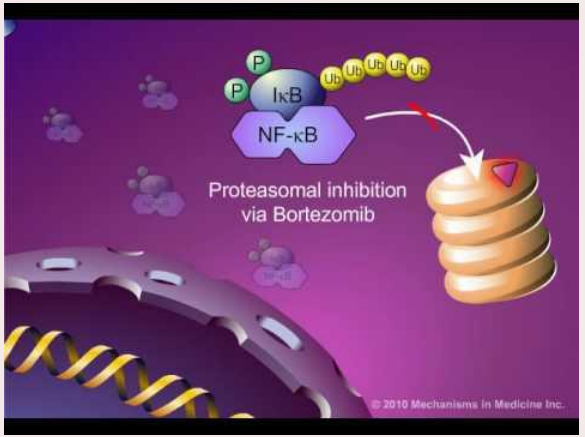
In patients with immune-mediated glomerular injury, the activation of NFκB has been shown by immunohistochemistry.
NFκB is overexpressed in IgA nephropathy, especially in patients with high proteinuria and reduced renal function
All these morphological examinations in human biopsy describe the correlation of NFκB expression and clinical outcome parameters in these patients
Enhanced glomerular NF-κB activity in human chronic kidney disease lead to increased risk of lupus-related GN

Kassan M et al. Enhanced NF-kappaB activity impairs vascular function through PARP-1-, SP-1-, and COX-2-dependent mechanisms in type 2 diabetes. *Diabetes*. (2013) 62:2078–87.
Herrington FD, Carmody RJ, Goodyear CS. Modulation of NF-kappaB signaling as a therapeutic target in autoimmunity. *J Biomol Screen.* (2016) 21:223–42.
López-Franco O et al. Nuclear factor-kappa B inhibitors as potential novel anti-inflammatory agents for the treatment of immune glomerulonephritis. Am J Pathol. 2002 Oct;161(4):1497-505.

Chemical structures of NF-kB inhibitors. (A) Ectinascidin 743; (B) Bortezomib; (C) Chromomycin A3; (D) Emetine; (E) Daunorubicinum; (F) Lestaurtinib.



Biochem Pharmacol. 2010 May 1; 79(9): 1272–1280.



Qayum A, Aleem A, Al Diab AR, Niaz F, Al Momen AK. Rapid improvement in renal function in patients with multiple myeloma and renal failure treated with bortezomib. Saudi J Kidney Dis Transpl. 2010;21(1):63-8.

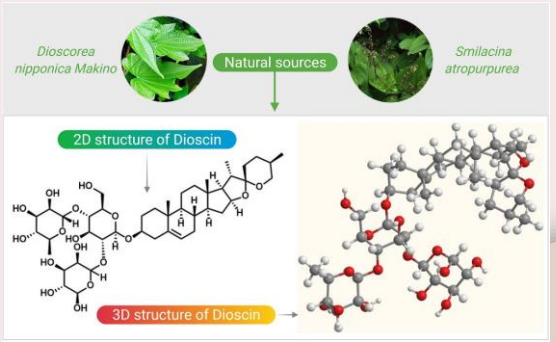
Experimental models

- It has been shown that NF-κB inhibitors **attenuate the induction of renal inflammation and injury in animal models**

A role of celastrol, an NFκB inhibitor, on insulin resistance and renal function has been demonstrated in diabetic *db*/*db* mice, where celastrol treatment reduced the creatinine levels, albuminuria, glomerular matrix expansion

Peritoneal injection of miR-451 improved renal function in diabetic nephropathy, and the effect was accomplished by the suppression of the LMP7/NFκB pathway-mediated proinflammatory molecule expression

Downregulation of NFκB can ameliorate kidney injury through the reduction of the glomerular NLRP3 inflammasome in diabetic nephropathy



Wang Y et al. Mechanism of dioscin ameliorating renal fibrosis through NF-κB signaling pathway-mediated inflammatory response. Mol Med Rep. 2023 Apr;27(4):93.

Kim JE et al. Celastrol, an NF-kappaB inhibitor, improves insulin resistance and attenuates renal injury in db/db mice. PLoS ONE. (2013) 8:e62068doi: 10.1371/journal.pone.0062068

Conclusion

- Obtained results may suggest that CNA may act as the DAMPs, probably bridging the kidney damage and immune cells activation via NF-kB activation
- No response has been documented for IRF3 and IRF7 for CKD, except for Transplanted patients