Susceptibility of Complement system proteins to citrullination with human PAD4

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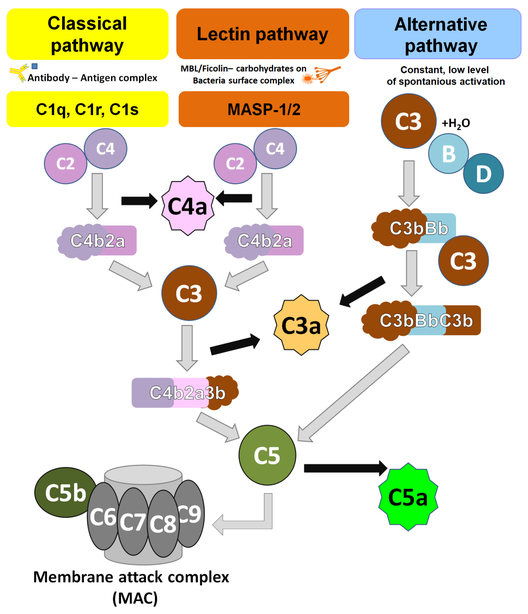
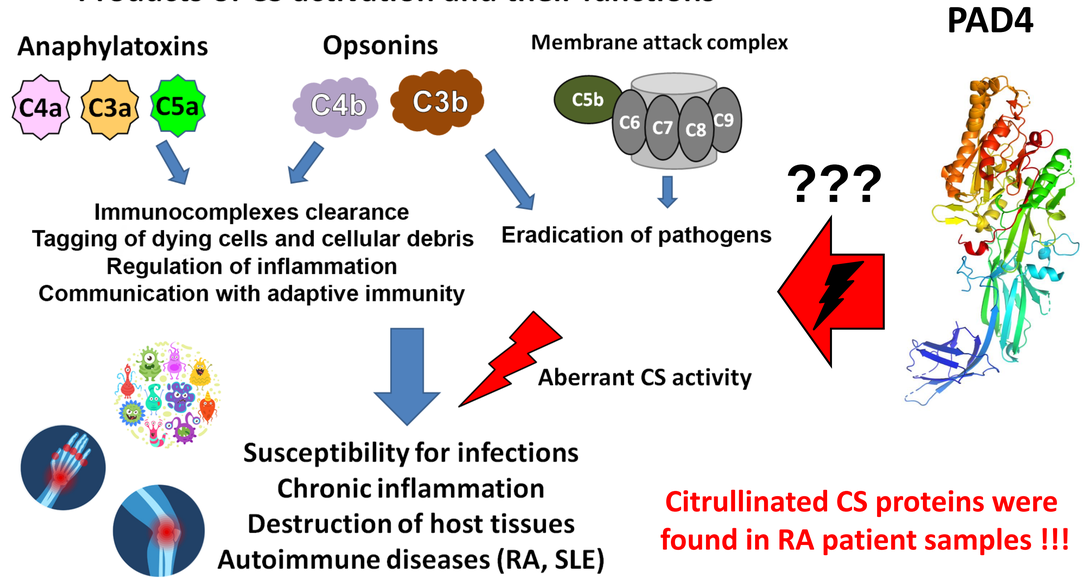
INTRODUCTION: The complement system (CS) is an essential element of the innate immunity and one of the primary defense systems against pathogens. However, the function of CS is not limited to the protection against invading microbes. Of equal importance are its capability of labeling apoptotic and necrotic cells and their fragments for effective clearance, the immunomodulatory potential in regulating the state of inflammation, and control of the interplay between the innate and adaptive branches of immune system. The disruption of the complement system homeostasis, both in its excessive or insufficient activation, leads to a number of pathological processes including development of the autoimmune diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE).

Protein citrullination and production of autoantibodies against citrullinated targets are hallmarks of rheumatoid arthritis. RA is also characterized by impaired activity of the complement system, including overactivation of proteins from the main cascade driven by immunocomplexes recognition, and insufficient activity of CS inhibitors. Broad proteomic analysis of citrullinome in samples of patients with RA revealed targets for PADs among complement proteins, but exact citrullination patterns, susceptibility of certain complement components and influence of deimination on their activity is not yet known.

Complement system cascade



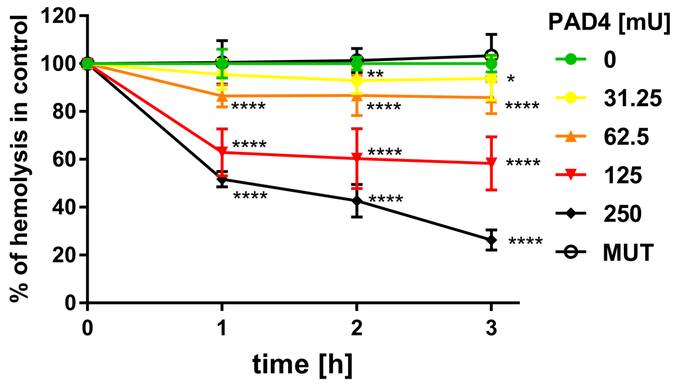
Products of CS activation and their functions



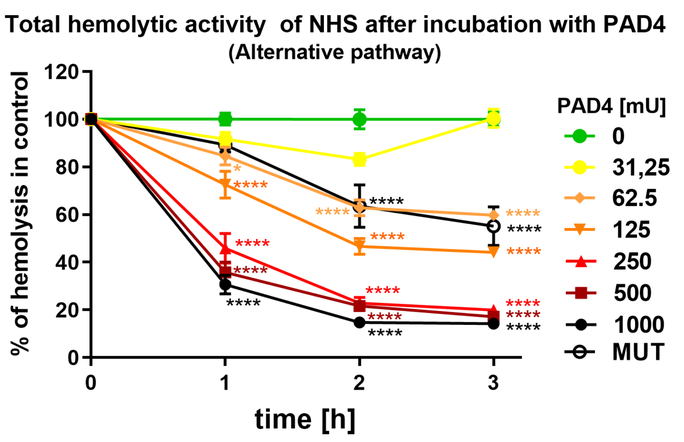
Citrullinated CS proteins were found in RA patient samples !!!

Citrullination with PAD4 decreases CS activity

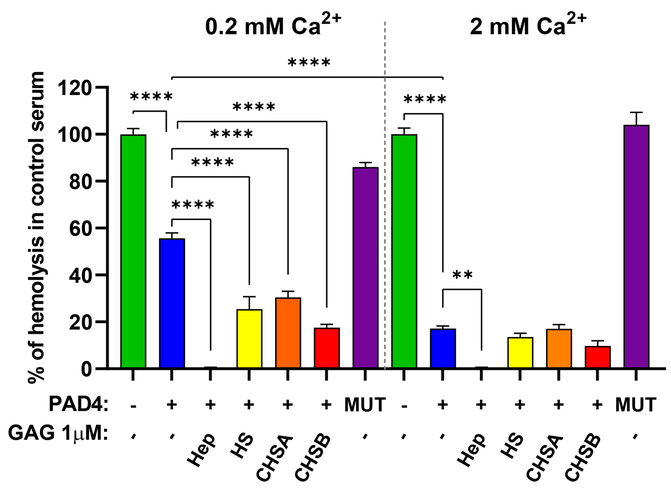
A Total hemolytic activity of NHS after incubation with PAD4 (Classical pathway)



B Total hemolytic activity of NHS after incubation with PAD4 (Alternative pathway)



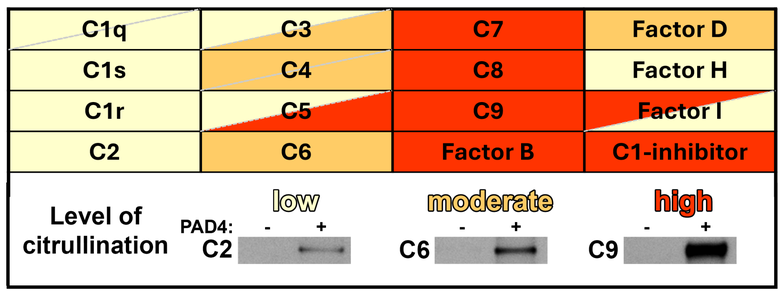
C GAGs increase PAD4 activity in low calcium concentration



▲ Total hemolytic activity of complement in normal human serum upon citrullination with PAD4 **(A)** Hemolytic assay for total complement activity in citrullinated NHS on Classical pathway with sheep erythrocytes sensibilized with anti-sheep erythrocyte IgG (activator of Classical complement pathway). **(B)** Hemolytic assay for total complement activity in citrullinated ΔC1q NHS on Alternative pathway with rabbit erythrocytes. **(C)** Hemolytic assay for total complement activity on Classical pathway with NHS citrullinated with PAD4 in two $CaCl_2$ concentrations in presence or absence of 1μM glycosaminoglycans (Hep – Heparin, HS – heparan sulfate, CHSA/B – chondroitin sulphate A/B). None of the GAGs in designated concentrations had any influence on NHS activity. Mut - catalytical mutant of PAD4. Results were calculated as % of untreated NHS control and are shown as mean +/- SEM.

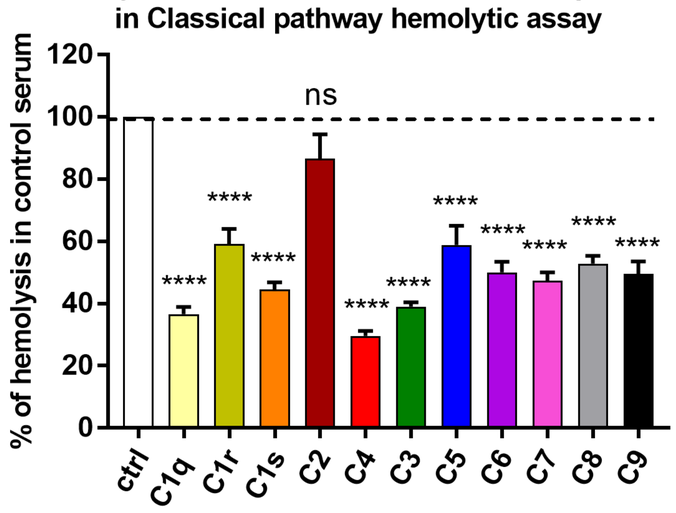
Major complement components are susceptible to citrullination *in vitro*

A

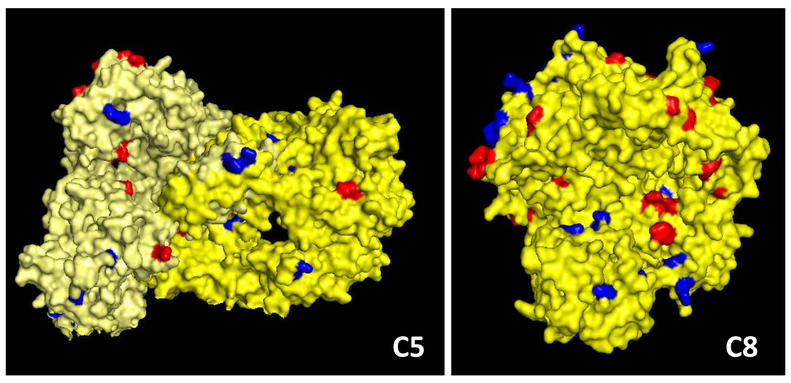


Citrulline specific probes – Bt-phenylglyoxal

B Activity of individual citrullinated CS components in Classical pathway hemolytic assay



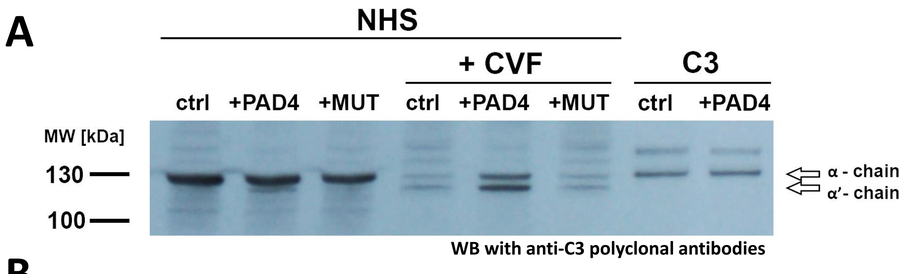
C



▲ **(A)** Exemplary results of Western blot with citrulline specific probes (Bt-phenylglyoxal) for three proteins - weakly citrullinated C2, moderately citrullinated C6, and strongly citrullinated C9. The intensity of citrullination for all 16 major complement proteins assessed with citrulline specific probes is shown as a heat map. Divided fields represent citrullination level of single chains within double-chained proteins. **(B)** Hemolytic assay for total complement activity on Classical pathway with purified native/citrullinated complement proteins with their corresponding depleted sera. **(C)** Crystal structures of C5 (PDB:3CU7) and C8 (PDB:3OJY) with marked arginine residues (**blue**) and citrulline residues (**red**) identified by MS analysis. Citrullination of complement system proteins seems to be selective as not all well-exposed arginin residues undergo modification.

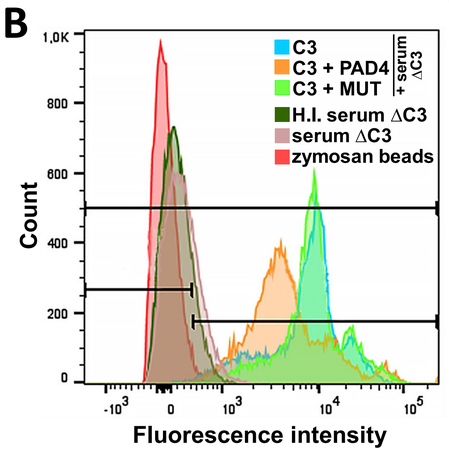
Citrullination of C3 influences its activation and processing

A



WB with anti-C3 polyclonal antibodies

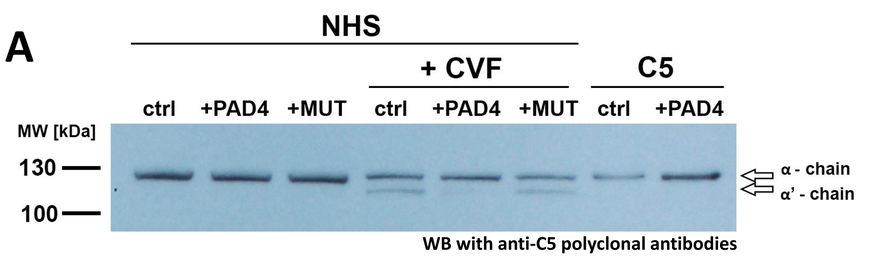
B



▲ **(A)** Analysis of C3 activation with Western Blot technique using anti-C3 polyclonal antibodies. Complement alternative pathway in the native/citrullinated serum was activated with Cobra venom factor (CVF) the analog of C3 convertase. Citrullination influences C3 activation and/or further processing upon activation. **(B)** Deposition of activated C3 fragments on zymosan coated beads measured with FACS. Fluorescently labeled C3 native or incubated with PAD4 was supplemented to ΔC3 human serum. Activation of the CS cascade was assesed on the surface of zymosan coated beads – activator of alternative pathway.

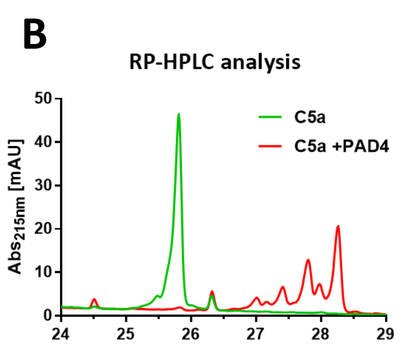
Citrullination modifies activation sequence of C5 and diminishes anaphilatoxic potential of C5a

A



WB with anti-C5 polyclonal antibodies

B



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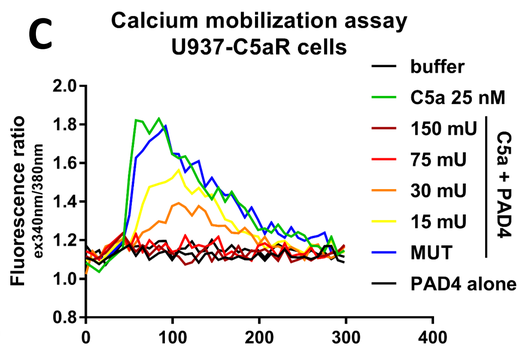

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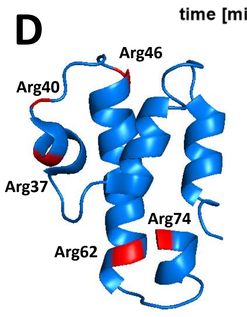

▲ **(A)** Activation of C5 was determined with Western Blot using anti-C5 polyclonal antibodies in native/citrullinated serum upon activation with CVF. Citrullination modifies C5 activation sequence and limits C5a release. **(B)** RP-HPLC analysis of native (**green**) and citrullinated (**red**) C5a indicating efficient modification of anaphylatoxin with PAD4. **(C)** Calcium influx assay on U937-C5aR cells. Cells loaded with Fura-2AM calcium detector were treated with 25 nM native or citrullinated C5a. Cell response to a test ligand was monitored with continuous fluorescence measurements at λ_{ex} = 340 nm and 380 nm and λ_{em} = 510 nm Values of fluorescence intensity for each time point were recalculated as the ratio of fluorescence intensity: λ_{em} = 510 nm at λ_{ex} = 340 nm to λ_{em}= 510 nm at λ_{ex} = 380 nm. (D) Structure of C5a with marked arginine residues which are modified by PAD4 based on MS analysis.

Conclusions

1. **Decreased activity of complement proteins upon citrullination is visible in both classical and alternative pathway.**
2. **Major CS components are susceptible for modification with PAD4, especially C3-C5 and MAC complex proteins which result in their decreased activity. Regulatory proteins like C1-INH, Factor B and Factor I are also extensively citrullinated.**
3. **Citrullination of C3 influence its activation and processing resulting in decreased opsonization potential.**
4. **Modification of C5 occurs within activation sequence of the protein resulting in limited activation with cobra venom factor.**
5. **Citrullination of C5a anaphylatoxin abrogate its activity and potential of immune cell stimulation. Moreover, deimination of C-terminal arginine in C5a is the first example of exodeiminase activity of human PADs.**

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