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Original Article

Decay-Accelerating Factor Differentially Associates With Complement-Mediated Damage in Synovium After Meniscus Tear as Compared to Anterior Cruciate Ligament Injury

V. Michael Holers [1], Rachel M. Frank [2], Michael Zuscik [2], Carson Keeter [2], Robert I. Scheinman [3], Christopher Striebich [1], Dmitri Simberg [3], Michael R. Clay [4], Larry W. Moreland [1,2], Nirmal K. Banda [1,*]

[1]Division of Rheumatology, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA
[2]Department of Orthopedics and the Colorado Program for Musculoskeletal Research, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA
[3]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA.
[4]Department of Pathology, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA

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***Correspondence to**
Nirmal K. Banda
Division of Rheumatology, School of Medicine, University of Colorado, Anschutz Medical Campus, 1775 Aurora Court, Aurora, CO 80045, USA.
Email: Nirmal.Banda@cuanschutz.edu

ORCID iDs
V. Michael Holers
https://orcid.org/0000-0002-5634-7746
Rachel M. Frank
https://orcid.org/0000-0002-1120-0521

ABSTRACT

We have reported that anterior cruciate ligament (ACL) injury leads to the differential dysregulation of the complement system in the synovium as compared to meniscus tear (MT) and proposed this as a mechanism for a greater post-injury prevalence of post traumatic osteoarthritis (PTOA). To explore additional roles of complement proteins and regulators, we determined the presence of decay-accelerating factor (DAF), C5b, and membrane attack complexes (MACs, C5b-9) in discarded surgical synovial tissue (DSST) collected during arthroscopic ACL reconstructive surgery, MT-related meniscectomy, osteoarthritis (OA)-related knee replacement surgery and normal controls. Multiplexed immunohistochemistry was used to detect and quantify complement proteins. To explore the involvement of body mass index (BMI), after these 2 injuries, we examined correlations among DAF, C5b, MAC and BMI. Using these approaches, we found that synovial cells after ACL injury expressed a significantly lower level of DAF as compared to MT ($p<0.049$). In contrast, C5b staining synovial cells were significantly higher after ACL injury ($p<0.0009$) and in OA DSST ($p<0.039$) compared to MT. Interestingly, there were significantly positive correlations between DAF & C5b ($r=0.75$, $p<0.018$) and DAF & C5b ($r=0.64$ $p<0.022$) after ACL injury and MT, respectively. The data support that DAF, which should normally dampen C5b deposition due to its regulatory activities on C3/C5 convertases, does not appear to exhibit that function in inflamed synovia following either ACL injury or MT. Ineffective DAF regulation may be an additional mechanism by which relatively uncontrolled complement activation damages tissue in these injury states.

Keywords: Complement system; Complement proteins; Decay-accelerating factor; Multiplexed immunohistochemistry; Anterior cruciate ligament; Medial meniscus tear; Lateral meniscus tear; Osteoarthritis; Synovium

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Michael Zuscik
https://orcid.org/0000-0003-0461-8708
Carson Keeter
https://orcid.org/0009-0001-2709-7338
Robert I. Scheinman
https://orcid.org/0000-0001-8485-6620
Christopher Striebich
https://orcid.org/0000-0003-2582-7752
Dmitri Simberg
https://orcid.org/0000-0002-5288-6275
Michael R. Clay
https://orcid.org/0000-0003-1659-8927
Larry W. Moreland
https://orcid.org/0000-0003-4483-0359
Nirmal K. Banda
https://orcid.org/0000-0002-5291-5136

Conflict of Interest

V. Michael Holers: royalties, consulting income, stock and stock options in complement therapeutics companies. Nirmal K. Banda: Royalties, consultant with Annexon Biosciences, and patent for the treatment of inflammatory diseases using the antibody RNA conjugate (ARC), i.e., anti-C5aR1mAb - C5siRNA. Other authors declare no potential conflicts of interest.

Abbreviations

ACL, anterior cruciate ligament; AP, alternative pathway; BMI, body mass index; CFB, complement factor B; CFH, complement factor H; CFHR4, complement factor H related 4; CI, confidence interval; CP, classical pathway; DAF, decay-accelerating factor; DSST, discarded surgical synovial tissue; FAP-α, fibroblast activated protein-α; FLS, fibroblast-like synoviocytes; LMT, lateral meniscus tear; LP, lectin pathway; MAC, membrane attack complex; MCP, membrane cofactor protein; MIHC, multiplexed immunohistochemistry; MIRL, membrane inhibitor of reactive lysis; MMT, medial meniscus tear; MS, multiple sclerosis; MT, meniscus tear; OA, osteoarthritis; PNH, proximal nocturnal hemoglobinuria; PTOA, post traumatic osteoarthritis; RA, rheumatoid arthritis; scRNA-seq, single-cell RNA-sequencing.

Author Contributions

Conceptualization: Holers VM, Banda NK; Data curation: Banda NK; Formal analysis: Keeter C, Clay MR, Moreland LW, Banda NK; Funding acquisition: Frank RM, Banda NK; Investigation: Banda NK; Methodology: Scheinman RI, Simberg D, Clay MR, Banda NK; Supervision: Banda NK; Validation: Frank RM, Striebich C, Banda NK; Visualization:

INTRODUCTION

Post traumatic osteoarthritis (PTOA) develops after joint injuries and affects ~5M people in the US each year (1,2). PTOA is often triggered by sports-related knee injuries, especially in young people, and it is also common in combat-injured soldiers. Anterior cruciate ligament (ACL) injury and meniscus tear (MT) are the 2 most common sports-related injuries which can lead to the early onset of PTOA (3-6). At present, surgical interventions are the best approaches for the repair of knee joint trauma and internal tissue disruption. Clinical benefits of reconstructive surgery and meniscectomy are significant, but still about 50% of patients with ACL and MT injuries develop PTOA (7) that demonstrates macroscopic inflammation. The reasons for the development of such synovial inflammation are incompletely understood.

Activation of the complement system, a serum effector system, has been shown to potentially associate with the development of PTOA due to its apparent activation after both ACL injury and MT (8). The complement system is activated by 3 pathways: classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). One end-product of the CP, LP and AP is the membrane attack complex (MAC), which is able to cause cellular injury and inflammation in various diseases. The level of activation of the complement system cascade is controlled by the relative activities of proteins that act as either activators or regulators. Complement fragments such as C3a, C3b and C5a, after complement activation, fuel effector function causing tissue damage in disease. In contrast, complement regulators work to inhibit complement activation in the fluid phase as well as on the cell surface. Four plasma membrane bound complement regulatory proteins are expressed specifically to protect the host cells from complement activation: CD59 (aka membrane inhibitor of reactive lysis [MIRL]), CD35 (aka type 1 complement receptor), CD46 (aka membrane cofactor protein [MCP]) and decay-accelerating factor (DAF) (9). The role of complement regulators in the development of PTOA after ACL and MT injury has been largely unstudied. Herein we explore the expression and potential roles of DAF in these processes.

DAF is a glycophosphatidylinositol anchored membrane protein broadly found on most cells and tissues, including erythrocytes, lymphocytes, granulocytes, endothelium and epithelium (10), and the presence of soluble DAF has also been reported in saliva, urine, plasma, tears and synovial fluid (11). In addition to being a complement regulatory protein, DAF binds CD97, a G protein coupled receptor (12,13). Upregulation of DAF on fibroblast-like synoviocytes (FLS) increases the binding capacity for CD97 (14). DAF functionally accelerates the decay of the CP convertase C4b2a (15), the AP convertase C3bBb (16) and also C5 convertases (17) by dissociating them into their constituent proteins and/or blocking their assembly (12,18), but does not exhibit cofactor activity for the factor I-mediated proteolytic degradation of C4b or C3b (16). These convertases are necessary for the formation of the MAC (19). A number of human diseases are associated with DAF deficiencies or altered functions. Some, like cancer, upregulate DAF to protect against complement mediated lysis (20). Others, like multiple sclerosis (MS) and proximal nocturnal hemoglobinuria (PNH), are amplified by DAF deficiency or relative decreases (20).

The role of DAF in rheumatoid arthritis (RA) is complex. It is expressed in a subset of FLS found in the synovial lining (21,22). DAF expression has been associated with arthritis progression in inflammatory RA mouse models (22,23) while the opposite was found for an immune-complex RA mouse model (22). DAF has been shown to be expressed at similar levels on FLS isolated from patients with RA, osteoarthritis (OA), psoriatic arthritis and

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spondyloarthritis (14). Interestingly, the level of expression of DAF may be regulated by engagement of pattern recognition receptors such as Toll-like receptor-3 and retinoic acid-inducible gene-I (14). Only one study examined the presence of DAF gene expression in human tissue after ACL injury in four phases before reconstructive surgery (21); however, no study has compared the presence of DAF in the synovial tissue after ACL injury and MT.

Previously, we found that ACL injury, more than MT, is associated with an increased deposition of complement C4d on the injured synovial cells, along with an increased dysregulation of the AP, while increased infiltration of mast cells in the synovium with MT is found as compared to ACL injury (8). Thus, MT and ACL could be the representations of a subacute inflammation, and an acute inflammation respectively. The main goal of this current cross-sectional study was to examine, compare and assess the potential biological significance of DAF expression using multiplexed immunohistochemical (MIHC) analysis along with C5b expression in parallel, in discarded surgical synovial tissue (DSST) after ACL injury and MT, and compare with OA DSST and control DSST. Our hypothesis is that DAF, independent from body mass index (BMI) and before reconstructive surgery and meniscectomy, might be playing an important role in the synovium after ACL injury and MT, which might lead to the accelerated development of PTOA.

MATERIALS AND METHODS

DSST from ACL, MT, and OA patients

DSST was obtained during ACL reconstruction and medial meniscectomy or medial meniscus repair in ACL or MT patients, respectively, as described previously (8). For this study, 6 to 8 DSST were obtained per patient with 9 related to ACL reconstruction, 12 related to MT meniscectomy, 4 related to OA (grade 4) before total knee replacement surgery, and 3 control cadaveric donors. The size of each DSST ranged from 0.5 mm to 3 mm (8). DSST were collected ~30–35 days post injury according to an Institutional Review Board, University of Colorado Anschutz Medical Campus approved protocol number 1056. All DSST from ACL injury, MT and OA were fixed in 10% neutral buffered formalin and embedded in paraffin wax followed by sectioning and staining with H&E for histopathology and synovial sections were also processed for complement protein MIHC staining and followed imaging analysis (8). All DSST from ACL and MT were also stained with Vimentin and examined by a pathologist for structural synovial integrity as described previously (8). All semiquantitative histological studies including vascularity, adipocyte numbers, fibroblasts and synovial membrane thickness using H&E staining of ACL, MT, OA and control DSST have already been published separately in detail (8). Two sections from limited number of ACL and MT DSST were used for estimating Krenn's and synovial numerical inflammation scores (24). Controls sections on slides similarly fixed in formalin and paraffin embedded, have no history of opportunistic infections, RA and OA and were commercially obtained (Articular Engineering, Northbrook, IL, USA). These synovial tissues were harvested ~6–8 h after death from cadaveric donors. Serology tests from these control donors were negative for HIV-1, Hepatitis C antibody, and Hepatitis B antigen (Articular Engineering). For this study, no distinction was made between medial meniscus tear (MMT) and lateral meniscus tear (LMT) patients.

MIHC of DSST for complement proteins

To determine the expression of DAF positive synovial cells, an antigen retrieval was performed with 10 mM Sodium Citrate pH 6.0 with incubation for 10 min at 110°C followed

by cooling for 10 min at room temperature. The primary rabbit monoclonal DAF antibody (Abcam, Cambridge, UK) used was 1:200 and incubated for 16 min at 37°C. The detection of DAF was done using the UltraView Universal detection kit as described previously (8). Similarly, to determine the expression of C5b positive synovial cells, an antigen retrieval with Borg solution pH 9.5 (BioCare Medical, Pacheco, CA, USA) was used. The dilution of C5b antibody used was 1:200 followed by incubation for 32 m at 37°C on a benchmark XT IHC Stainer. To examine the presence of fibroblast in the DSST after ACL injury and MT, rabbit monoclonal antibody to fibroblast activated protein-α (FAP-α), SP325 (Abcam) (dilution 1:100, antigen retrieval EDTA buffer, Leica, ER2) was used. To examine the presence of macrophages in the DSST after ACL injury and MT, a mouse monoclonal antibody with reactivity to human CD68 (Dako, Carpinteria, CA, USA) (dilution 1:500, antigen retrieval citrate buffer, Leica ER1) was used. The counterstain was done with Harris Hematoxylin according to previously described methods (8). For DAF, C5b, FAP-α and CD68 detection, Ventana UltraView DAB universal polymer detection from Ventana/Roche, using the Ventana Benchmark XT, was used according to published methods (8). We have used various human tissue (surgical discard) such as RA synovium, placenta, liver and human cerebellum (brain), tonsil and kidney to examine the specificity of DAF, C5b, FAP-α and CD68 antibodies. DAF, C5b, FAP-α and CD68 related MIHC staining were performed using the Akoya Biosciences detection kit and multispectral imaging using the Vectra Polaris systems. MIHC images from ACL, MT, OA and control DSST were scanned to obtain the percentage of synovial cells expressing DAF, and C5b complement proteins. Similarly, all the MIHC composite images from the DSST after ACL injury and MT were also scanned to obtain percentage of synovial cells expressing DAF, FAP-α and CD68 in the synovial lining or synovial membrane and sub synovial lining area.

Quantitative measurements of complement proteins in DSST

All stained sections were scanned followed by imaging to determine the expression of DAF and C5b in the DSST. After scanning and imaging of MIHC images from ACL, MT, OA and control synovium, all data were exported from the image analysis software (InForm, Los Angeles, CA, USA) according to our published studies (8,25). The frequency of positive cells for DAF and C5b from each synovial section was calculated by dividing the number of cells with positive staining by the total number of cells in each region, according to our published studies (8). The software used identifies each individual cell using DAPI staining nuclei. A threshold is set for DAF and C5b depending on the range of signals seen across the images. The percentage of cells that are above the threshold are considered positive and quantified. A false color-coding key was used to identify DAF (yellow) and C5b (green) positive synovial cells in the DSST.

We also quantified by using an algorithm the percentage of macrophages and fibroblasts in the synovial membrane expressing DAF on their surface.

Statistical analyses

The mean differences between the percentage of DAF and C5b positive synovial cells from the ACL, MT, OA and control DSST data obtained from MIHC quantitative imaging analysis were compared by using both student *t*-test and Mann-Whitney test. The normality of data was checked using the Kolmogorov-Smirnov test. In some analysis, based on the nature of data, a 2-way ANOVA Bonferroni test or an Unpaired *t*-test with Welch's correction was used. Multivariate regression analysis was used to explore the relationship between BMI (as a dependent variable), DAF and C5b (both as independent variables). To find correlations

Table 1. Characteristics of ACL and MT synovial tissue patients

DSST	ACL	MT
Number	9	12
Age (yr)	33.33±11.99	49.50±10.32
Sex (female)	6 (66.6)	11 (91.6)
Race (Caucasian)	7 (77.8)	10 (83.3)
Ethnicity (non-Hispanic)	9 (100.0)	11 (91.6)
BMI (kg/m^2)	25.09±4.13	34.02±22.84 (p<0.261)
Orientation (right)	7 (77.8)	7 (58.3)

Values are presented as mean ± SD or number (%).

among various variables DAF, C5b, MAC, Krenn's scores and BMI, Pearson correlation was used. All data were plotted using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as mean ± SEM, with p-values <0.05 considered significant.

RESULTS

Characteristic of ACL and MT subjects, and processing of DSST

Demographic characteristics of subjects with ACL injury and MT from whom DSST were obtained by an orthopedic surgeon has been shown in **Table 1**. Characteristics of each subject including sex, age, BMI, race, ethnicity, and the limb (right or left) from which DSST was obtained are also shown in **Table 1**. Additional detailed characteristics of subjects including specific diagnosis, and surgical procedure were published in our previous study (8). All MIHC studies were conducted blindly.

MIHC of DSST for DAF

To explore why ACL inflammation is more severe than MT, we compared using MIHC, the expression of DAF in the DSSTs (**Fig. 1**). We found that ACL DSST expressed a significantly (p<0.049) lower level of DAF on synovial cells compared with MT DSST (**Fig. 1**). Interestingly using multivariate regression analysis, we noticed that on average, DAF was 14 units larger in the MT group when compared to the ACL group (95% confidence interval [CI], 6.3–21). The correlation (r=0.55) between DAF and histopathological Krenn's scores in ACL DSST was positive but non-significant (p<0.29). Similarly, the correlation (r=0.44) between DAF and Krenn's scores in MT DSST was positive but nonsignificant (p<0.45). However, OA DSST expressed significantly higher levels of DAF compared to MT DSST (p<0.016) and ACL DSST (p<0.014) (**Fig. 1**). The percentage of synovial cells expressed DAF in control DSST was minimal i.e., 1.0±0.343. A representative composite image of MIHC showing the presence of DAF (yellow color) has been shown using a false color-coding scheme (**Fig. 2A and B**). Comparatively, a higher DAF expression in the ACL and MT DSST was predominantly noticed in the synovial membrane and was present at higher levels in the MT DSST synovial membrane (**Fig. 2A and B**). No major visual differences were seen in the cellularity of the 1–2 cell layer constituting synovial membrane comparing ACL DSST and MT DSST. Visually, DAF was present more in the sub synovial lining area and around the adipocytes in the MT DSST compared with the ACL DSST (**Fig. 2C and D**). These data overall suggest that after ACL injury, there may be more complement activation and less regulation due to the relative absence of DAF.

MIHC of DSST for C5b expression

To further confirm that ACL injury impacts the synovium more severely in ACL injury than the MT, we examined the expression of C5b using MIHC, which is an initial component of the MAC assembly on the surface of cells (**Fig. 3**). ACL DSST expressed significantly

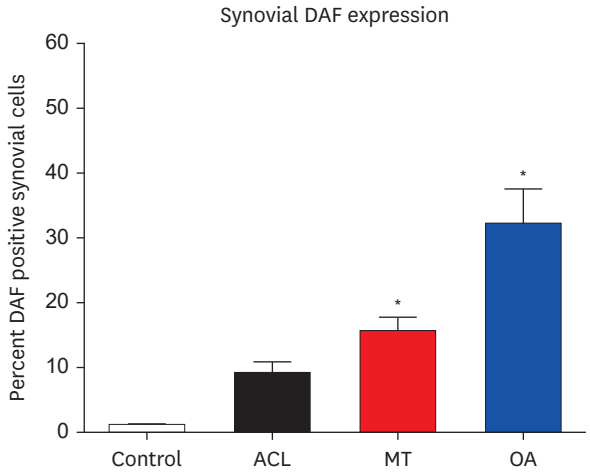

Figure 1. Comparing using MIHC percentage of synovial cells expressing DAF and showing representative composite images of DAF presence in the DSST. Percentage of DAF positive ACL synovial cells (black color) and MT synovial cells (red color) at the time of reconstructive surgery and meniscectomy, respectively. Expression of DAF from control DSST (no color) and OA DSST (blue color) is also shown. ACL DSST: n=9. MT DSST: n=12. Control DSST: n=3 and OA DSST: n=4. In OA DSST, DAF expression was significantly higher than MT DSST and ACL DSST. In MT DSST, DAF expression was significantly higher than ACL DSST. All DSST were included in the final analysis for reproducibility and variability. ANOVA with the Dunn-Bonferroni *post hoc* analysis was used to find out the differences. Data are shown as mean ± SEM.
*p<0.05 considered significant.

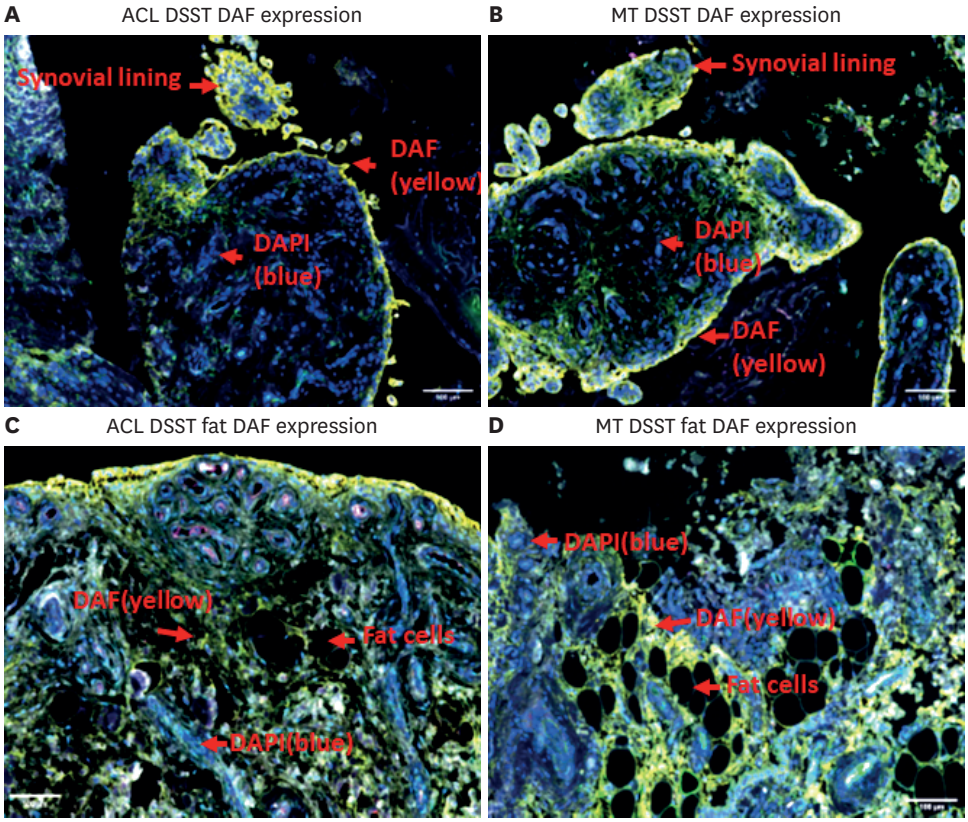

Figure 2. Representative composite images of MIHC for the percentage of synovial cells differentially expressing DAF from ACL injury and MT. (A) A compositive image from ACL synovium showing presence of less DAF (yellow color) in the synovial lining and sub-synovial lining area. (B) A composite image from MT synovium showing presence of more DAF (yellow color) in the synovial lining and sub-synovial lining area. (C) Presence of less DAF around the adipocytes in the sub-synovial lining area in ACL synovium (D) Presence of more DAF around the adipocytes in the sub-synovial lining area in MT synovium. False color-coding keys include yellow = DAF and DAPI = blue. Adipocytes = black cobble-like stone cells in synovium. A red arrow shows the presence of DAF positive synovial cells. A minimum of 3 composite images were generated using a single DSST with 2 sections from each ACL (n=9) and MT (n=12) patient. Scale bar=100 μm.

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(p<0.009) higher levels of C5b in the synovium compared to MT DSST (**Fig. 3A**). We found statistically significant evidence that there is a linear trend between DAF and C5b (p=0.005) in the ACL and MT DSST. For every 1-unit increase in C5b, DAF increases by 30 units (95% CI, 10–49). The correlation (r=0.55) between C5b and synovial inflammation scores in ACL DSST were positive but not significant (p<0.24). The correlation (r=0.22) between C5b and synovial inflammation scores in MT DSST were also positive but nonsignificant (p<0.66). Similarly, OA DSST expressed significantly (p<0.039) higher levels of C5b compared to MT DSST (**Fig. 3A**). C5b expression was also present in the sub synovial lining areas in ACL and MT DSST (**Fig. 3B and C**). In control DSST a minimal percentage of synovial cells, i.e., 2.5±1.19 expressed C5b on their surface (**Fig. 3A**). A representative composite image of C5b (green color) from ACL and MT DSST is shown (**Fig. 3B and C**). These data suggest that ACL injury potentially impacts the synovium more compared with the MT through increased C5b activation and deposition functioning to catalyze MAC formation.

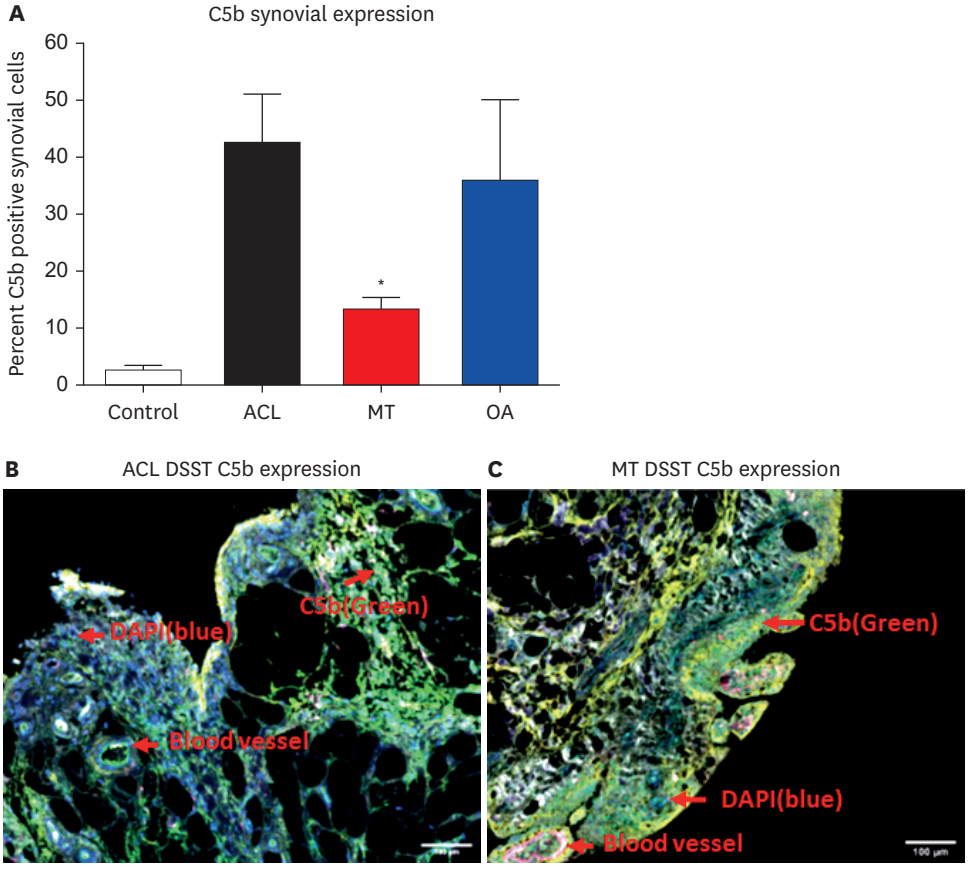

Figure 3. Comparing complement C5b expression on the synovium after ACL and MT injury and also composite images from MIHC showing the presence of C5b on synovial cells. (A) Percentage of C5b positive ACL synovial cells (black color) and MT synovial cells (red color) at the time of reconstructive surgery and meniscectomy, respectively. Expression of C5b from control DSST (no color) and OA DSST (blue color) is also shown. ACL DSST: n=9. MT DSST: n=12. Control DSST: n=4 and OA DSST: n=4. All DSST were included in the final analysis for reproducibility and variability. In MT DSST a significantly lower C5b expression was seen compared with ACL DSST and OA DSST. ANOVA with post-hoc analysis was used to find out the differences. Data are shown as mean ± SEM. (B) A composite image from synovium after ACL injury showing the presence of more C5b (green color) predominately in the sub synovial lining area and inside blood vessels. (C) A composite image from synovium after MT showing the presence of less C5b (green color) predominately in the synovial lining and sub synovial lining area also some in the blood vessels. False color-coding keys include green = C5b, and DAPI = blue. A red arrow shows the presence of C5b positive synovial cells. A minimum of 3 composite images were generated using a single DSST with 2 sections from each ACL (n=9) and MT (n=12) patient. Scale bar=100 μm.
*p<0.05 considered significant.

BMI of study subjects

To explore if there are any differences in the BMI of subjects from which DSST were obtained after ACL and MT injury, we compared the BMI with the understanding that it may or may not be affecting the complement proteins. First, we found that there were no significant ($p<0.261$) differences in the BMI in the subject with ACL injury (33.33±11.99) and MT (49.5±10.32) (**Table 1**). Additionally, there are no correlations among BMI, DAF and C5b-9 data sets (data not shown). Second, using Multivariate regression, we found no statistically significant evidence that there is a trend between DAF and BMI ($p=0.8$). These data confirm that a lower level of DAF expression but a higher expression of C5b noticed in the DSST after ACL injury are related to the specific impact of these injuries on synovium but not to the BMI.

Percentage of DAF expressing cells in the synovial lining and sub synovial lining area after ACL injury and MT

To find out what specific cells are present in the synovial lining or intimal lining and sub synovial lining area after ACL injury and MT in the DSST and if there are any differences in the expression of DAF on their surface, we quantified the percentage DAF expressing macrophages and FAP-α positive cells from composite images after MIHC (**Fig. 4**). We found that the percentage of macrophages and fibroblasts in the synovial lining or synovial membrane after ACL injury were 17.81±5.36 and 6.41±2.29 respectively (**Fig. 4A**). While the percentage of macrophages and fibroblasts in the synovial membrane after MT were 4.08±1.68 and 0.744±0.533, respectively (**Fig. 4A**). The percentage of both macrophages and fibroblasts were significantly ($p<0.05$) higher in the synovial membrane after ACL injury than the MT ($p<0.05$). The percentage of macrophage expressing DAF in the synovial membrane after ACL injury and MT were 5.64±2.27 and 3.95±1.49 respectively (**Fig. 4A**). On the other hand, the percentage of fibroblasts expressing DAF in the synovial membrane after ACL and MT were 4.11±1.53 and 0.543±0.459, respectively (**Fig. 4A**). In the sub synovial lining area, in the MT DSST, the percentage of DAF expressing were more and significant ($p<0.030$) compared with ACL DSST (**Fig. 4B**). Interestingly the percentage of FAP expressing cells was more and significant ($p<0.030$) in the sub synovial lining area in the ACL DSST than the MT DSST (**Fig. 4B**). Consistently we found that there were more cells in the synovial lining and sub synovial lining areas expressing DAF after MT than after ACL injury (**Fig. 4A and B**). These MIHC data also show that besides macrophages and fibroblasts there are other unknown synovial membrane cells which express DAF on their surface after ACL injury and MT. Representative composite images of the synovial membrane cells expressing DAF have been shown for ACL and MT respectively has been shown (**Fig. 4C and D**).

Correlations among DAF, C5b and MAC in the DSST after injury

We compared the correlation between synovial cells expressing DAF and C5b in the DSST after ACL injury and MT (**Fig. 5**). We found that there was a positive and significant ($r=0.75$, $p<0.018$) correlation between the proportion of ACL DAF and ACL C5b expressing synovial cells (**Fig. 5A**). Similarly, there was a positive and significant ($r=0.64$, $p<0.022$) correlation between MT DAF and MT C5b expressing synovial cells (**Fig. 5B**). These data suggest that DAF increases on the synovial cells with synovial inflammation or complement activation after both ACL injury and MT. Nonetheless a lower expression of C5b in the MT synovium confirms that the impact of injury is less as compared to ACL injury. To further analyze this feature, we found a negative but non-significant correlation between DAF expression and MAC expression on the synovial cells after ACL injury ($r=-0.49$, $p<0.17$) (**Fig. 6A**). In contrast, the correlation between DAF expression and MAC expression on the synovial cells after MT was positive but still non-significant by 2 tail *t*-test ($r=0.54$, $p<0.067$) however significant by

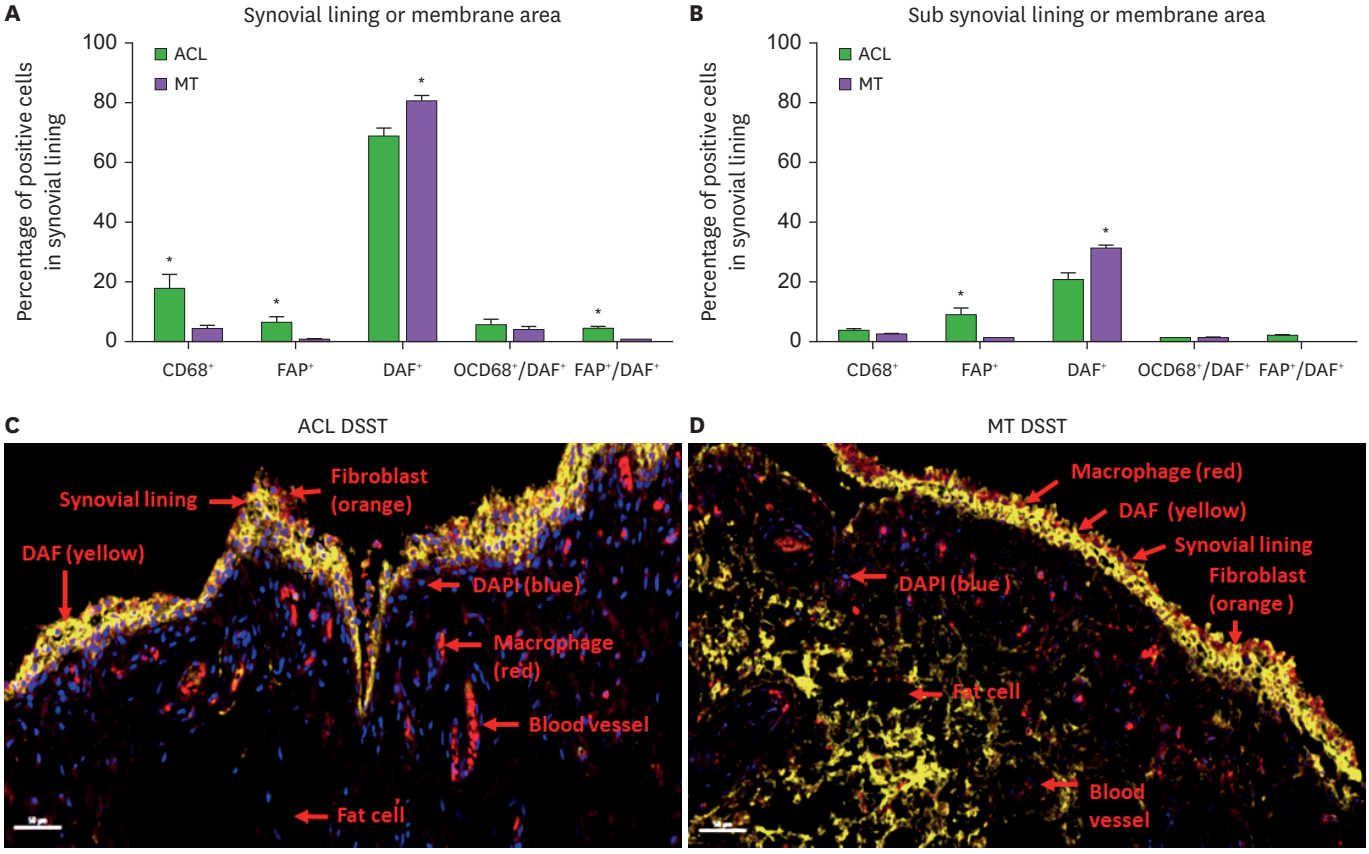

Figure 4. A bar graph and representative of composite images comparing the percentages of DAF expressing cells in the synovial membrane or lining and sub synovial lining area from DSST after ACL injury and MT. (A) Graph showing the percentage of single and double positive cells, i.e., macrophage and fibroblasts expressing DAF on their surface in the synovial lining from the DSST after ACL injury and MT. (B) Graph showing the percentage of single and double positive cells, i.e., macrophage and fibroblasts expressing DAF on their surface in the sub synovial lining area from the DSST after ACL injury and MT. (C) A composite image from ACL synovium lining showing presence of macrophage (red color)/DAF$^+$ (yellow color), fibroblasts (orange color)/DAF$^+$ (yellow) cells in the synovial lining. (D) A composite image from MT synovium lining showing presence of macrophage (red color)/DAF$^+$ (yellow color), fibroblasts (orange color)/DAF$^+$ (yellow) cells in the synovial lining. False color-coding keys include yellow = DAF, macrophage = red, fibroblast = orange and DAPI = blue. Adipocytes = black cobble-like stone cells in synovium. A red arrow shows the presence of DAF$^+$/macrophages$^+$ and DAF$^+$/fibroblast$^+$ synovial cells. The *t*-test was used to compare the percentages of DAF expressing single and double positive cells in the synovial membrane. A minimum of 3 composite images were generated using a single DSST with 2 sections from each ACL (n=4) and MT (n=4) patient. Magnification 20×. Scale bar=50 μm.

one tail *t*-test (**Fig. 6B**). MAC values used for these correlations were published previously and matched with the subjects (8). We also found that the mean ratios of DAF$^+$: C5b$^+$ synovial cells in ACL DSST were less than 1, i.e. 0.23 while the mean ratio of DAF$^+$: C5b$^+$ synovial cells in MT DSST were 2.43, i.e., 2.0. Similarly, the mean ratios of DAF$^+$: MAC$^+$ synovial cells in ACL DSST were less than 1, i.e., 0.22 while the mean ratios of DAF$^+$: MAC$^+$ synovial cells in MT DSST were 4, i.e., 4.2. These differential ratios confirm that ACL is a more severe injury than MT and might be the representation of acute inflammation and subacute inflammation respectively. Once again, these correlations are consistent with the hypothesis that DAF is ineffectively protecting the synovium from complement activation after MT and ACL injury, though differential regulation of the formation of MAC relative to C5b is present in the two disease states.

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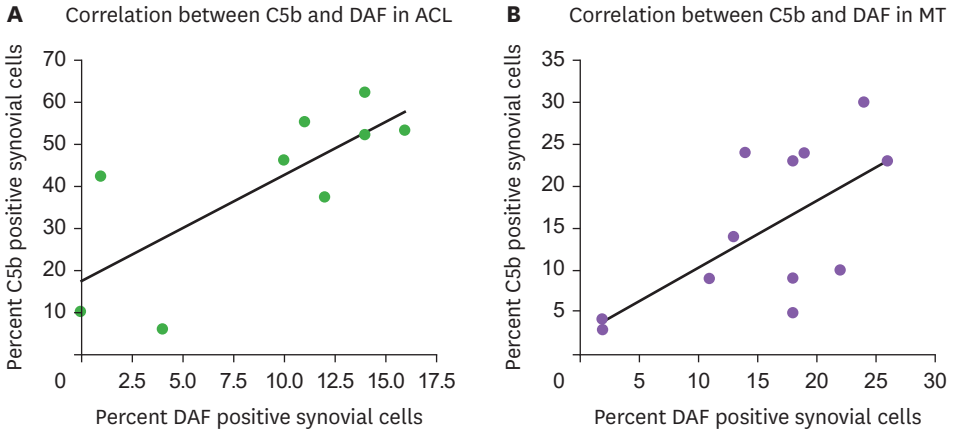

Figure 5. Correlation between DAF and C5b in the synovium after ACL and MT injuries. (A) A positive correlation between percentage of DAF and C5b expressing synovial cells after ACL injury. (B) A positive correlation between percentage of DAF and C5b expressing synovial cells after MT. ACL: n=9 and MT: n=12.

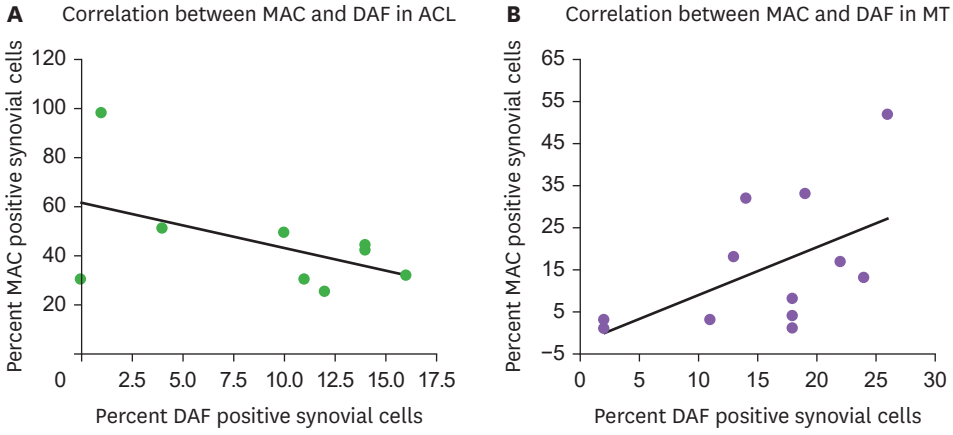

Figure 6. Correlation between DAF and MAC in the synovium after ACL and MT injuries. (A) A negative correlation between the percentage of DAF and MAC expressing synovial cells after ACL injury. (B) A positive correlation between percentage of DAF and MAC expressing synovial cells after MT. ACL: n=9 and MT: n=12.

DISCUSSION

There are several relevant findings in this current study using DSST after ACL and MT injuries but before arthroscopic ACL reconstructive surgery and MT-related meniscectomy. First, synovial cells expressed a lower level of DAF on their surface after ACL injury compared to MT and OA. Second, synovial cells expressed a high level of C5b after ACL injury compared to MT. Third, DAF expression was not related to patient BMI. Fourth, there were positive correlations between the expression of DAF and C5b after ACL injury and MT on the synovial cells, suggesting that DAF expression increases with inflammation, injury and complement activation. In addition, there was a negative but non-significant correlation between DAF and MAC after ACL injury compared to a positive but non-significant correlation between DAF and MAC after MT. Overall, all data supports that DAF, which should normally dampen C5b deposition due to its conventional regulatory activities on C3/C5 convertases, although expressed differentially relative to C5b and MAC, does not appear to appropriately exhibit full inhibitory function locally in inflamed synovia following either ACL injury or MT. The low expression of DAF on synovial cells in the DSST from ACL injury vs. MT patients confirms that this injury impacts the synovium more severely than MT, likely in part through increased

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complement activation. The decrease in DAF protein expression noticed in ACL synovium could be due to shedding of DAF under acute inflammatory conditions but might not be due to under-expression at a transcriptional or protein level. Previous studies have also shown that cytokines such as IL-4 and IL-1β lead to shedding of DAF from cell membrane surfaces in human intestinal epithelial cells, HT-29 (26). Furthermore, actinomycin D, cycloheximide and brefeldin A decreased the expression and release of DAF from the cell surface induced by IL-4 and IL-1β (26). Other studies have shown that TGF-β1, TNF-β, IFN-γ increased expression of DAF on ocular fibroblasts in contrast to MCP and MIRL or CD59 (27). Thus, due to an altered cytokine environment locally in the joints after ACL injury, i.e., acute inflammation vs. MT injury, subacute inflammation there might be an excessive shedding of DAF after ACL injury. Therefore, cytokines could differentially suppress the function of DAF in the synovial membrane after ACL injury and MT.

DAF is known to inhibit complement activation by interfering with the functions of C3 and C5 convertases in both CP and AP (11,15,16), which generate proinflammatory mediators including C3a and C5a after cleaving C3 and C5, respectively. Thus, low levels of DAF would be expected to correlate with poor control of complement activation after ACL injury and MT. Little to no DAF or C5b was found in synovium from uninjured control tissues in contrast to OA synovium confirming DAF's role during inflammation or injury. Interestingly, DAF levels correlate with C5b levels both in synovial tissue from ACL DSST (**Fig. 5A**) and from MT DSST (**Fig. 5B**). Despite the increase in ACL patients, DAF induction is less able to keep up with C5b generation as compared to MT patients. We have reported a high expression level of MAC in the synovial tissue using DSST after ACL injury compared to MT (8). C5b is formed through C5 cleavage and initiates MAC on the surface of cells, with subsequent lysis and/or damage to the target cells. C5b is expressed more than 3-fold on the synovium after ACL injury compared to MT (**Fig. 3A**). The mechanism why there is an increased expression of DAF in the synovial lining both in ACL and MT DSST? It could be due to altered cytokine expression or levels after these injuries in the knee joints. A spike in cytokine expression has been reported in the synovial fluid after ACL injury and MT (28,29). DAF expression as well as of other complement inhibitors such as CD46 and CD59 in a human hepatocyte cell line, Hep3B increased in the presence of TNF-α, IL-6 and IL-1β to protect it from complement mediated attack (30). Since all DSST from ACL injury and MT were examined for DAF expression after ~30–35 days post injury therefore prolonged time of DAF expression could be due to altered cytokine expression to protect from sustained complement attack on synovial cells in the joint after these injuries. More in-depth studies related to the cytokine expression and of DAF along with complement proteins of the terminal pathways at the single-cell RNA-sequencing (scRNA-seq) are needed to examine the correlation between DAF and various complement split fragments. From these data, we conclude that the fundamental processes eliciting DAF regulation are still present and intact after these injuries but fail to protect from developing PTOA.

We asked if there are similarities between overexpression of DAF and IL-1Ra in an acute injury such as ACL but insufficient to protect and override the cytokine storm? IL-1Ra, a natural antagonist of IL-1 cytokine, prevents the activity of IL-1α and IL-1β. Studies have shown that the expression of IL-1Ra1, a receptor for IL-1Ra increased on human OA chondrocytes and synovial fibroblasts in comparison with normal cells (31,32). IL-1β acts as a mediator of cartilage destruction in OA. Low grade (subclinical) inflammation results in synovial synovitis in early stages of OA (33). A variety of cytokines and chemokines such as IL-1β, TNFα, IL-6, IL-8, IL-15, and IL-17 have been reported to be involved in promoting synovitis (33,34). Nonetheless, IL-1β and TNF-α, were considered major players in OA pathogenesis

because injection of both of these cytokines into the knee joints of rabbit caused cartilage destruction (35). We have also shown the role of complement proteins such as C4d in the inflammatory events that lead to the development of PTOA (8). It is a well-accepted concept that DAF protects human cells from an autologous complement attack (36). These authors have also shown that DAF is expressed on the surface of resting neutrophils and the surface expression more than doubles when cells activated (36). Low and high expression of DAF after ACL injury and MT respectively, might indicate that synthesis and translocation of DAF to the synovial cell surface after these injuries and DAF might be helpful to the survival of synovial cells in the synovial membrane to maintaining the first line of defense in an active inflammatory microenvironment where there might be a cytokine storm and, also a huge complement turnover due to ongoing low-grade inflammation. We have shown that there is an increased expression of C4d (cellular) (significant) and C3c, C3d (cellular) (non-significant) on the surface of synovial cells from ACL injury than the MT (8), therefore, presence of C4b or C3b on synovial cells in a cytokine rich environment and their interactions with DAF can't be ruled out. DAF is known to interact with surface bound C4b and C3b therefore inhibits the assembly of CP and AP C3 and C5 convertases. Decreased expression of DAF on ACL synovial DSST shows a rapid turnover or shedding probably due to the acute nature of ACL injury compared with MT. Thus, one can draw inferences that DAF presence on the synovial lining could be similar to the role of IL-1R1 in OA where its constant presence is needed to inhibit complement and have effect on disease.

These data are consistent with our previously published observations that ACL injury impacts the synovium more due to more C4d deposition and complement dysregulation due to an increased expression of complement factor H related 4 (CFHR4) on synovial cells. Again, this was observed more in ACL DSST than the MT DSST and was proposed as one of the reasons, independent from BMI, for the increased development of PTOA after ACL injury than MT (8). Our current data show that after ACL injury and MT there is excessive activation of the complement system because of C5b component mediated activation of the terminal pathway of complement system despite a high level of DAF, which has also been found in OA DSST. The specific pathway activating complement in these injuries remains unknown. Expression of DAF in the synovial lining in OA DSST is consistent with the previous reports in OA and RA (23). Previously, we and others, have suggested a key role for the complement protein C5 in humans and in mouse models of PTOA (37).

We confirmed the presence of macrophages and fibroblasts in the synovial lining and sub synovial lining areas after ACL injury and MT (**Fig. 4**). Also, the synovial lining was intact in all ACL and MT DSST (**Fig. 4A and B**). One of the interesting observations was that specifically in the synovial lining and in the sub synovial lining area after ACL injury than the MT, percentage of fibroblasts expressed more DAF while there were no differences in the percentage of macrophages expressing DAF on their surface. In contrast, overall synovial lining from MT DSST expressed more DAF than the ACL DSST indicating some unknown cells express DAF beside macrophages and fibroblasts. Again, these data confirm the differential impact of these 2 injuries on complement inhibitors, immune and inflammatory cells in the synovial membrane and in the sub synovial areas. Previously we have already shown the presence of mainly macrophages and synovial fibroblasts in the synovial membrane from patients with RA (25). Therefore, these 2 types of cells are the most likely DAF expressors in the synovial membrane, but we don't rule out there might be other synovial membrane mesenchymal stem cells expressing it. DAF has been shown to be generated by FLS and deposited on the local collagen fiber meshwork where it protects the

synovial tissue from immune-complex-mediated arthritis (22). Alternatively, we do not know if there is a relationship between attenuated DAF expression in the DSST in current study and increased CD8 T cells after ACL injury compared to MT, which we have reported previously (8). One previous study using DAF deficient mouse has shown that DAF suppresses T cell immunity *in vivo* (38). We also do not rule out the protective role of DAF in the sub-synovial lining area. Also, DAF is expressed more around the adipocytes in the synovium after MT than the ACL injury (**Fig. 2D**) with speculation that some adipokines generated by the adipocytes might be influencing the expression of DAF in the vicinity.

We also have re-analyzed our previous data (8) with matching patients to the current study to explore if there are any correlations among DAF, and other complement proteins; C3c, complement factor B (CFB) and complement factor H (CFH). We found no correlations between ACL DAF vs. ACL C3c, CFB, and between MT DAF vs. MT C3c, CFB and there were no significant correlations (data not shown). Furthermore, the correlation between synovial membrane thickness and DAF expression from available samples was negative and positive in ACL and MT DSST respectively (data not shown). Since we do not find any above-mentioned correlations between DAF, Krenn's scores, inflammation scores and major complement fragments or components locally along with synovial membrane thickness in the DSST obtained from knee joints after ACL and MT therefore we cannot speculate or make a statement whether any of these components generated locally or systemically.

We do recognize some limitations in our study. First, we have limited number of available DSST after ACL injury and MT and could not analyze DAF expression data separately from male and females. Second, we have not identified various types of immune and inflammatory cells other than macrophages and fibroblasts present in the synovial membrane expressing DAF on their surface because we have not standardized the MIHC staining and antigen retrieval conditions for other markers like stromal cell-derived factor 1 (CXCL12), HAS1 and, PRG4 etc. Therefore, we can't say whether macrophages or intimal lining synovial fibroblast expressed or synthesized DAF or that soluble DAF (made elsewhere) simply binds to CD97 on yet unidentified synovial lining cells. CD97 has been reported to be present on the surface of activated leukocytes, for example (13). Third, to explore excessive shedding or consumption of DAF from synovial membrane after ACL injury than MT in parallel evaluation of the synovial fluid collected at the time of surgeries will be valuable to estimate the absolute levels of soluble DAF. Unfortunately, we did not have access to synovial fluid along with DSST when these studies were conducted. ScRNA-seq and Cellular Indexing of Transcriptomes and Epitopes by Sequencing studies using synovial cells will be more informative to differentiate an inadequate DAF gene expression from excessive consumption after ACL injury than the MT and these studies are in progress. Thus, low expression C5b and high expression of DAF in the synovial tissue after MT might be playing a protective role to delay the development of PTOA in contrast to ACL injury. Further studies related to the DAF and C5b protein expression in synovium associated with synovial inflammation score are needed based on various immune cells and inflammatory cells infiltrates.

The clinical relevance of current study derives from the positive correlation of DAF with C5b after ACL and MT. DAF-like therapeutics such as the fusion protein, TT32, which is a potent inhibitor of both CP and AP (39), could be useful for repeated injections after ACL injury locally in the knee joints.

In summary, in this cross-sectional study, we report a connection between DAF and C5b expression in the synovium after ACL and MT injuries as a potential pathogenic driver for the development of PTOA. DAF is distinctly and specifically localized in the proximity of synovial membrane (**Fig. 2**) after MT and ACL injury, and it might be protecting synovial membrane immediately after injury. Why DAF fails to protect synovium after these injuries might to due to an increase in other CFH counter regulatory proteins such as CFHR4 locally creating an imbalance (8)? Nonetheless the immunosuppressive potential of DAF in the synovial membrane can't be ruled out after these injuries. DAF has also been associated with many diseases such as angiopathic hyperactivation, complement hyperactivation (40), malaria (41), glomerular diseases (42), MS (43), RA (44), PNH (45), vitiligo (46), bullous pemphigoid (47), and cancer (48). PNH erythrocytes lacking DAF and CD59 are highly susceptible to MAC-mediated lysis and a mAb to C5, Eculizumab can be used for its treatment. Collectively our current study improves our understanding regarding the differential role of DAF in 2 different injuries, i.e., ACL and MT, which can differentially lead to the development of one PTOA. Thus, insufficient expression of DAF or its ineffective regulation in the synovium might lead to an abnormal complement activation and to an early development of PTOA after ACL injury and MT. Therefore, our study offers unique opportunities to use complement inhibitors immediately after ACL injury and MT to block complement activation and MAC formation.

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