A Novel 4-Dimensional Live-Cell Imaging System to Study Leukocyte-Endothelial Dynamics in ANCA-associated Vasculitis

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Abstract

Neutrophils, monocytes and the endothelium are critical to ANCA-associated vasculitis (AAV) pathogenesis. This study aimed to develop a 4-dimensional (4D) live-cell imaging system that would enable investigation of spatial and temporal dynamics of these cells in health and disease. We further aimed to validate this system using autologous donor serum from AAV patients and polyclonal ANCA IgG, as well as exploring its potential in the pre-clinical testing of putative therapeutic compounds. Neutrophils and monocytes were isolated from peripheral venous blood of AAV patients or healthy controls and co-incubated on an endothelial monolayer in the presence of autologous serum. Alternatively, polyclonal ANCA IgG was used, following TNF-α priming, and imaged in 4-dimensions for 3 hours using a spinning disc confocal microscope. Volocity 6.3® analysis software was used for quantification of leukocyte dynamics. The use of autologous serum resulted in increased neutrophil degranulation ($P = 0.002$), transmigration ($P = 0.0096$) and monocyte transcellular transmigration ($P = 0.0013$) in AAV patients. Polyclonal MPO-ANCA IgG induced neutrophil degranulation ($P < 0.001$) in this system. C5aR1 antagonism reduced neutrophil degranulation ($P < 0.0002$). We have developed a novel 4D *in vitro* system that allows accurate quantification of multiple neutrophil- and monocyte-endothelial interactions in AAV in a single assay. This system has the potential to highlight dynamics key to pathophysiology of disease, as well investigating the impact of potential therapeutics on these functions.

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1. Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) is a group of devastating autoimmune disorders characterised by relapsing necrotising vasculitis of small blood vessels, commonly involving kidneys and lungs. Considering the aggressive inflammation and multisystem nature of AAV, it is associated with significant morbidity and mortality [1]. The aetiology of AAV is unclear, but genetic and environmental factors, including infections, are proposed in the initiation of autoimmunity through dysfunctional innate and adaptive immune responses [2]. ANCA autoantibodies develop in susceptible individuals and target the antigens myeloperoxidase (MPO) and proteinase 3 (PR3). These antigens are present in the azurophilic granules of neutrophils and lysosomes of monocytes [3].

ANCA activate neutrophils and monocytes *in vitro* and contribute to the pathogenesis of AAV through inappropriate activation of several innate immune mechanisms including degranulation, cytokine production and generation of reactive oxygen species [4,5]. *In vivo* studies have further supported a role for ANCA in AAV pathogenesis [6–8]. Passive transfer of MPO-ANCA into recombinase-activating gene-2-deficient mice resulted in the development of features of AAV including pauci-immune glomerulonephritis, granulomatous inflammation and haemorrhagic pulmonary capillaritis [6]. Persistent inflammation in AAV causes upregulation of adhesion molecules on leukocyte and endothelial cell surfaces, facilitating the migration and transmigration of leukocytes into neighbouring tissue. Leukocyte degranulation then occurs and damages the endothelium due to release of toxic enzymes and this cycle continues, resulting in end organ damage [9–11]. This process is further driven in response to chemotactic factors, such as complement component 5a (C5a), which has been shown to have a prominent role in AAV pathophysiology [12,13]. In addition, recent clinical trial data showed that C5a receptor inhibition was effective in replacing high dose glucocorticoids in AAV [14].

Traditionally *in vitro* studies have focused on single cell populations [4,15,16], particularly neutrophils, and often restricted to study limited number of readouts. This prevents individual cellular influences to be quantified accurately. Sophisticated *in vitro* studies are invaluable in examining the contribution of interactions between leukocytes and endothelium, or other cells e.g. platelets, towards disease initiation and progression. In this study, we aimed to develop a 4D system that would allow investigation of these spatial and temporal dynamics of monocyte- and neutrophil-endothelial interactions in leukocytes isolated from patients with AAV. This, for the first time, would allow multiple features of AAV pathophysiology to be examined in a co-culture assay. We then aimed to validate this system using autologous patient serum, or polyclonal ANCA IgG, to highlight potential pathophysiologic differences. We hypothesise that ANCA IgG will show increased degranulation and transmigration in neutrophils and monocytes compared to anti-GBM and control IgG. Finally, we investigate the impact of C5aR antagonism on key leukocyte dynamics as an example of how this system could be employed to test therapeutic compounds.

2. Materials and Methods

2.1 Patients and Samples

This study was conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions under the ethical approval of the North of Scotland Research Ethics Committee REC reference 13/NS/0028. AAV patients were identified via the Vasculitis Clinic at Aberdeen Royal Infirmary. Following informed consent, peripheral venous blood was collected from 25 AAV patients with a diagnosis of either Granulomatosis with Polyangiitis (GPA), Microscopic Polyangiitis (MPA) or Eosinophilic Granulomatosis with Polyangiitis (EGPA) according to Chapel Hill consensus definitions [17] and 16 healthy donors for investigation of autologous serum and leukocytes. To investigate the role of complement C5aR1, 6 active AAV patients were recruited (supplementary material Table 1). Polyclonal ANCA IgG experiments utilised only healthy donor cells (n = 12). Vasculitis activity was measured using Birmingham Vasculitis Activity Score (version 3) [18] and clinical data was obtained for all patients, including AAV diagnosis, disease duration, ANCA status, organ involvement, C-reactive protein levels, estimated glomerular filtration rate, creatinine levels and treatments.

2.2 Leukocyte Preparation

Immediately following collection of blood, fresh peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on Lymphoprep^{TM} (Axis-Shield, Norway), followed by positive selection of $CD14^+$ monocytes using the MACS system (Miltenyi Biotec, Germany), according to manufacturer's instructions.

Neutrophils were isolated by density centrifugation using Histopaque 1119/1077 (both Sigma, Dorset, United Kingdom).

Leukocytes were fluorescently labelled with carbocyanine dyes 1,1'-Dioctadecyl-3,3,3',3'tetramethylindodicarbocyanine 4-chlorobenzenesulfonate (DiD) or Vybrant 3,3′dihexadecyloxacarbocyanine perchlorate (DiO) (2.5µl) (Molecular Probes, Invitrogen, Massachusetts, United States), re-suspended at a density of 1.5 x 10^6 each in CO_2-independent medium containing either 10% volume/volume autologous serum alone or plus 25 nM selective C5aR1 inhibitor, NDT 9513727 (Tocris Bioscience, Abingdon, United Kingdom) vs control dimethyl sulfoxide (DMSO). For polyclonal ANCA IgG experiments, leukocytes were isolated from healthy controls (HCs) and stained as above, primed with 2ng/ml TNF-α for 20 min at 37°C. Primed leukocytes were incubated with 200 µg/ml, plasma protein G purified, PR3-ANCA or MPO-ANCA to represent AAV disease or control IgG which included anti-GBM, ANCA-negative, transplant patients and healthy donors, throughout the 3 hour imaging assay. The polyclonal IgG identity was blinded until post analysis. 5µg/ml monoclonal MPO-ANCA (Clone 2C7, Origene) was incubated with TNF-α primed neutrophils to induce degranulation and supernatant collected to quantify lactoferrin by sandwich ELISA (Abcam, UK) (supplemental data Figure 2) to confirm visualisation of degranulation in the *in vitro* system.

2.3 Endothelial Cell Culture

Adherent HUVEC-C cell line C (American Type Culture Collection (ATCC), Manassas, Virginia, USA) were seeded into ‘µ-slide 2 well glass bottom dish’ (Ibidi, Munich, Germany) > 24 hours prior to imaging to form a confluent monolayer. HUVEC-C were labelled with 5µM CellTrace Violet (Molecular Probes, Invitrogen, Massachusetts, United States) before imaging with neutrophils and monocytes in the presence of CO2-independent medium containing 10% volume/volume autologous donor serum, 200 U/ml penicillin-streptomycin antibiotics and 2 mM L-glutamine (Invitrogen, Paisley, United Kingdom).

2.4 Image Processing and Data Collection

Neutrophils, monocytes and HUVEC were then immediately imaged following labelling using an Ultra Vox spinning disc confocal with a Yokogawa® CSU-X1 and additional micro lens spinning disc in the co-culture with HUVEC-C for 3 hours. Volocity 6.3 (Perkin Elmer, Massachusetts, United States) imaging analysis software was used to quantify the leukocyte functions including degranulation, migration and transmigration. Distinction between paracellular or transcellular routes of transmigration can also be quantified (supplemental data Figure 1).

2.5 Statistical Analysis

Statistical analysis was performed using Graphpad Prism software version 7 (Graphpad Software Inc, La Jolla, CA, USA). All data was tested for Gaussian distribution using the D'Agostino and Pearson normality test. Patient versus healthy population data was tested for significance using unpaired t-test (parametric) or Mann-Whitney test (non-parametric). Comparison of more than 2 data sets were analysed for using a One-way ANOVA. Actual statistical tests used will be stated throughout.

3. Results

3.1 Development of 4-Dimensional Imaging System

In order to investigate leukocyte-endothelial dynamics in 4D we developed a system that allows quantification of leukocyte-endothelial dynamics relevant to the pathophysiology of AAV. This required fluorescent labelling of leukocyte and endothelial cell membranes with fluorophores that were bright at low concentrations, low photo-toxicity, non-transferrable and quick staining protocols in order to minimise handling of cells (as detailed in Section 2).

Use of sophisticated image analysis software, Volocity 6.3, in combination with advanced microscopy technique, allowed combined and/or independent visualisation and quantification of leukocyte-endothelial cell dynamics. For example, this system can be visualised as a whole 4D image (Figure 1A; supplementary material Movie 1), or specific functions can be focused on. Leukocyte degranulation (Figure 1B/1D; Movie 2 and 3) can be quantified visually. We stimulated with monoclonal MPO-ANCA, a potent inducer of degranulation, and then correlated with lactoferrin, a traditional biochemical marker of degranulation, to confirm that this system accurately represented this dynamic (supplemental data Figure 2). The system can also investigate adhesion, migration, transmigration (Figure 1C) and route of transmigration (transcellular or paracellular) [Figure 1 near here].

3.2 Validation of the 4D Imaging System using Autologous Serum

To validate this *in vitro* system we recruited 17 active and 8 remission AAV patients, plus 16 healthy donors. Clinical features and immunosuppressive treatments of AAV patients are summarised in Table 1. We then incubated AAV patient or healthy donor leukocytes with a HUVEC-C monolayer in the presence of autologous serum. In these experiments neutrophil degranulation was significantly higher in the active AAV patient cohort compared to HCs (P =

0.0002) (Figure 2A). Active AAV patient monocytes had similar rates of degranulation compared to HCs (P = 0.0910) (Figure 2B). We then compared leukocyte degranulation in patients with active disease (BVAS > 0) with those in remission (BVAS = 0). A significantly higher neutrophil degranulation was observed in those with active disease (P = 0.0028) (Figure 2C), but no difference was detected in monocyte degranulation (data not shown). Both monocytes and neutrophils from active AAV patients and HCs had a similar mean migration over 3 hours (supplementary material Table 2). Neutrophils from AAV patients had significantly higher rates of transmigration than HC neutrophils (P = 0.0096) (Figure 3A). The percentage of the monocyte population that transmigrated the endothelial cell layer demonstrated no significant difference between active AAV and HCs (P = 0.0907) (Figure 3B). Neutrophils from AAV patients showed a preference for transcellular transmigration, although this was not statistically significant (P = 0.0525) (Figure 3C). However, the AAV monocytes that did transmigrate indicated a significant preference for transcellular transmigration compared to HCs (P = 0.0008) (Figure 3D) [Table 1, and Figure 2 and 3 near here].

3.3 Polyclonal ANCA IgG stimulates leukocyte degranulation but not transmigration

We used protein G purified polyclonal PR3-ANCA, MPO-ANCA, or control IgG to further validate and highlight leukocyte-endothelial dynamics that may be ANCA-dependent in this system.

We demonstrated that MPO-ANCA, but not PR3-ANCA, stimulated neutrophil (P < 0.001) (Figure 4A), not monocyte (Figure 4B), degranulation compared to control IgG. There was no significant difference in migration (supplementary material Table 4) or any transmigration parameters (Figure 4C/D/E/F) of neutrophils or monocytes when stimulated with either MPO-ANCA, PR3-ANCA or control polyclonal IgG [Figure 4 near here].

3.4 C5aR1 Inhibition Reduces Neutrophil Degranulation

We used the C5aR1 antagonist, NDT 9513727, to explore the system's potential to study leukocyte dynamics in the presence of immunomodulatory agents. C5aR1 inhibition resulted in reduced neutrophil ($P = 0.0002$) (Figure 5A), but not monocyte (Figure 5B), degranulation in active AAV patients' samples compared to control DMSO. C5aR1 inhibition was associated with a decrease in monocyte ($P = 0.0440$), but not neutrophil, migration compared to control DMSO (supplementary material Table 3). Transmigration of neither neutrophils or monocytes from AAV patients were affected by C5aR1 inhibition, including total population percentage that transmigrated, the route or the total distance of transmigration (supplementary material Table 3) [Figure 5 near here].

4. Discussion

Numerous studies have investigated the role of leukocytes in AAV, however to our knowledge this is the first *in vitro* 4D live-cell imaging platform that encompasses several biological readouts into one assay, particularly within the context of this disease. The development of this system required staining of neutrophil, monocytes and endothelial cells in order to image live cells with minimal impact from phototoxicity. This has allowed us to quantify spatial and temporal dynamics of monocyte and neutrophil-endothelial dynamics, demonstrating altered functions between AAV patients and the healthy population. In addition, the use of C5aR1 antagonism validates the system as a useful tool for screening of therapeutic compounds through its ability to alter leukocyte dynamics.

The use of autologous serum in our study highlighted an increased propensity of AAV neutrophils to degranulate significantly more than healthy donors. Of further interest was the significantly increased degranulation of neutrophils in active patients compared to those in remission. This indicates that the presumed proinflammatory profile of the serum during active disease maintains its influence *in vitro*. Our results extends on the body of literature that neutrophil degranulation contributes to the pathophysiology of AAV [4,19]. Our imaging system has the advantage of quantifying degranulation in a co-culture system, unlike traditional biochemical assays that cannot distinguish between cell types. To our knowledge, there is no previously published data on degranulation of neutrophils, monocytes and endothelial cells in a co-culture. Therefore, our data may not be directly comparable to neutrophils or monocytes when assayed alone.

We showed that MPO-ANCA, but not PR3-ANCA, stimulated TNF-α primed neutrophil and monocyte degranulation significantly more than anti-GBM, or control IgG in neutrophils. MPO-ANCA has been shown to be a more potent stimulator of neutrophils *in vitro* [20] and has demonstrated greater success in development of animal models of the disease [21].

Polyclonal ANCA IgG has been used in previous studies to stimulate degranulation in neutrophils and monocytes [5,19,22] and was used as a method to validate this important dynamic in this system. The use of monoclonal ANCA would likely produce less variable results. This is because monoclonal antibodies have higher specificity for a single epitope, as opposed to polyclonal IgG, which recognises multiple epitopes [23,24]. It has been shown that ANCA IgG has different affinities [25,26] and avidities [27] and can result in altered clinical or *in vitro* phenotypes. This was further supported in a brief report from Popat and Robson [28] who recently suggested that polyclonal ANCA IgG does not consistently stimulate TNF-α primed neutrophils, echoing the findings from Franssen *et al*., [29], much to the contrary of abundantly published literature in the field.

In our system, the use of a C5a receptor antagonist, showed a significant reduction in the incidences of neutrophil degranulation, supporting a role for C5a in neutrophil activation and involvement in AAV pathogenesis [30,31]. More importantly, this result validates the ability of this imaging system to demonstrate manipulation of leukocyte dynamics through potential therapeutic interventions, serving as an important tool for future studies.

There is conflicting evidence on the role of ANCA on transmigration dynamics [32,33]. Our data demonstrated that active AAV neutrophils had an increased rate of transmigration compared to healthy donors in the presence of autologous serum but not with ANCA IgG. Of interest, was the significant preference for transcellular transmigration seen in active AAV monocytes. This pattern may be relevant for disease pathogenesis but needs to be replicated in other *in vitro* and *in viv*o studies to clarify. Previous studies suggested the contribution of dominant expression of adhesion molecules such as ICAM-1 and PECAM-1 in driving transcellular transmigration [34]. There was no difference in leukocyte transmigration dynamics in our system in the presence of C5aR1 inhibitor, despite C5a being known as a potent chemoattractant to both neutrophils and monocytes [35]. Often leukocyte transmigration

is measured using a Transwell® insert [36]. In our system, we quantify transmigration through visualisation of the XZ- or YZ-planes. This allows multiple metrics of transmigration to be quantified such as total population transmigrating, depth of leukocyte movement, as well as the route of transmigration i.e. paracellular or transcellular. A further improvement in transmigration rates may have occurred if HUVEC were primed with TNF-α prior to addition of autologous sera, as demonstrated during our polyclonal ANCA experiments. Furthermore, this system may be enhanced by the addition of flow or with an upper/lower chamber to more easily facilitate leukocyte movement and to allow the application of chemotactic agents, such as N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP).

Much of the literature in AAV focuses on the influence of purified ANCA on neutrophil dynamics but our data intimates that serum factors also play a crucial role in transmigration and degranulation independent of ANCA. This is similar to the findings of Kraaij *et al.*, [37] who reported that depletion of IgG from active AAV patient serum retained the ability to produce excessive NET formation, but contrary to our finding on degranulation, this was not dependent on C5aR. Our data demonstrated a high degree of heterogeneity across all populations. This may represent altered pro-inflammatory profiles due to the heterogeneous nature of the disease and the variation in clinical scores of patients recruited. An increased sample size, in addition to longitudinal data, may improve this. Furthermore, given the endothelial necrosis seen in AAV pathogenesis *in vivo*, a limitation of this system is that it highlights early events, but not the long-term effects, of serum or ANCA on endothelial health.

We have developed a 4-dimensional live-cell imaging system that can investigate key functions of leukocytes in the context of health and disease and can be used as a tool to investigate potential therapeutic targets. Furthermore, the system has the capacity to examine many other relevant pathological mechanisms including neutrophil extracellular trap (NET)-osis, micro-particle- and platelet-endothelial interactions. It is to be determined whether the multi-metric

co-culture dynamics of this 4D live-cell imaging system outweigh the use of multiple *in vitro* biochemical measurements. During characterisation of this system, our preliminary data demonstrates that neutrophils and monocytes likely contribute to the AAV pathophysiology through dominant mechanisms and that these functions in AAV are altered compared to healthy donor leukocytes.

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Table 1 - Patient Characteristics (autologous serum experiments)

Figure 1: 4-Dimensional Live Cell Imaging Model of ANCA-associated Vasculitis. A) Video 1 still image of 4D in vitro model of AAV. B) Video 2 still image depicting independent cell populations during analysis. C) XZ plane micrograph showing transmigration through endothelial cell layer. D) Video 3 still images of monocyte degranulation from 60-160 min showing sequential release of monocyte-derived particles subsequently seen around body of cell (scale bar = 25 μm). Monocytes (DiD - red); neutrophils (DiO - green); HUVEC (CellTrace Violet - blue). 4D – 4-dimensional; ANCA - anti-neutrophil cytoplasmic antibodies; AAV – ANCA-associated vasculitis; HUVEC – human umbilical vein endothelial cell

Figure 2: Leukocyte Degranulation. Data represents percentage of population exhibiting degranulation during 3-hour imaging assay with autologous serum. Degranulation of active AAV versus healthy donors in A) neutrophils (n = 16; 12) (P = 0.0002) and B) monocytes (n = 16; 13) (P = 0.0910). C) Neutrophils from active patients (n = 16) versus remission patients (n = 7) (P = 0.0028). Data shown is mean ± SEM. Data tested for Gaussian distribution. Unpaired t-test (A/B/C); ** P< 0.01; *** P<0.001.

Figure 3: Leukocyte Transmigration. Data represents percentage of population that transmigrate HUVEC during 3 hour imaging assay with autologous serum. A) Neutrophil transmigration (n = 16; 6) (P = 0.0096) B) monocyte transmigration (n = 16; 7) (P = 0.0907) C) Neutrophil transcellular (n = 16; 6)(P = 0.0525) D) monocyte transcellular (n = 16; 7)(P = 0.008). Data shown is mean ± SEM. Data tested for Gaussian distribution. Unpaired t-test; ** P< 0.01, *** P<0.001.

Figure 4: Polyclonal ANCA IgG. Data represents the percentage population of healthy donor TNF-α primed leukocytes exhibiting measured functions following stimulation with polyclonal PR3-ANCA (n = 9), MPO-ANCA (n = 11), or control IgG (n = 8). A) Neutrophil degranulation

438 (***P < 0.001) B) Monocyte degranulation C) Neutrophil transmigration D) Monocyte
439 transmigration E) Neutrophil transcellular route F) Monocyte transcellular route. Data shown
440 is mean ± SEM. Anti-GBM control IgG. Data tested for Gaussian distribution. One-way
441 ANOVA with Bonferroni multiple comparison test.

442 **Figure 5: C5aR1 Degranulation.** Inhibition of C5aR1 versus control DMSO (n = 6) A)
443 Neutrophils (P = 0.0002). B) Monocytes (P = 0.9560). Data shown is mean ± SEM. Paired t-
444 test *** P<0.001.

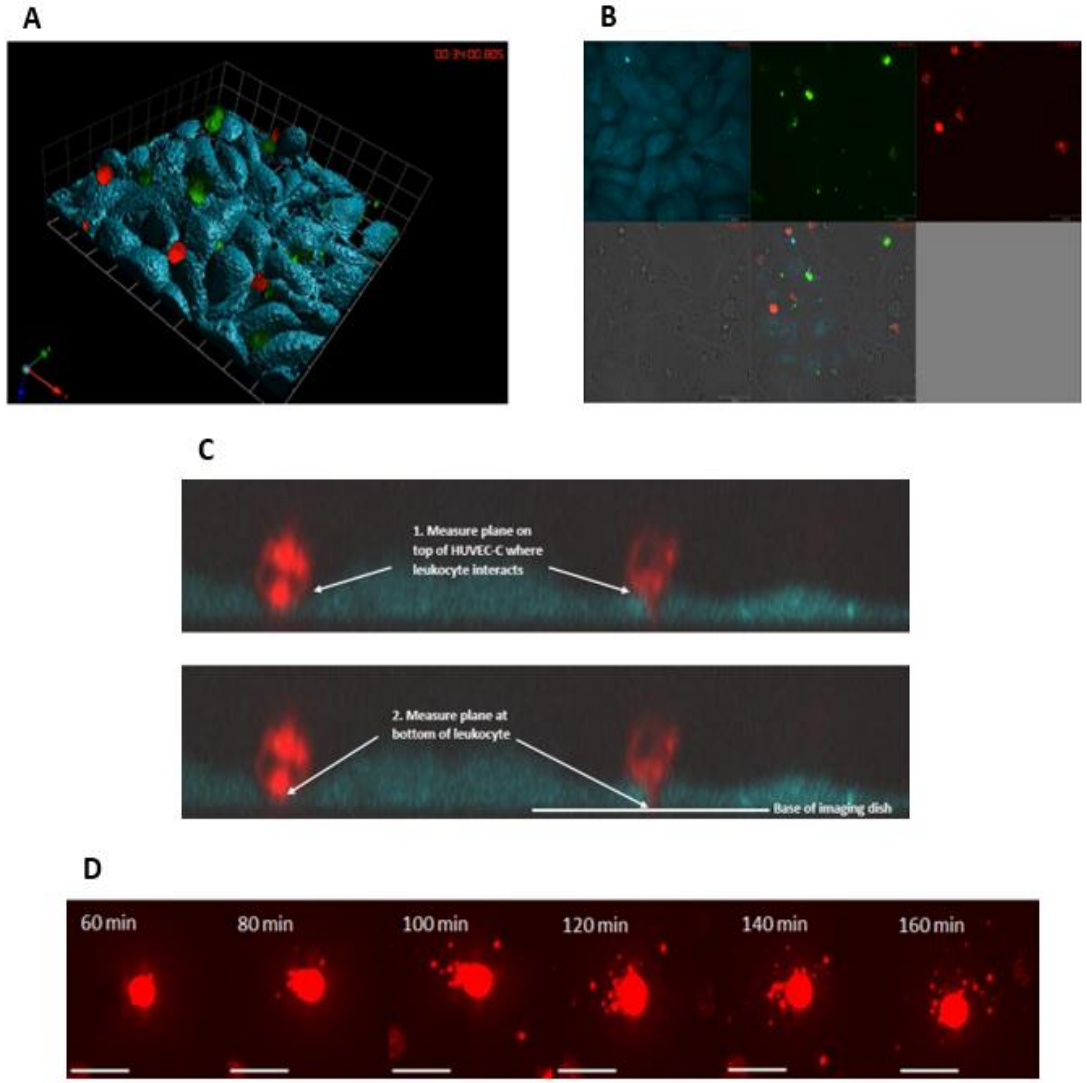
Table 1 Patient Characteristics (autologous serum experiments)

Variables			
	Active Patient (*n = 17*)	Remission Patient (*n = 8*)	Healthy (*n = 16*)
Age, median (range), years	59 (40-77)	62 (50-72)	43 (27-60)
Sex, male	10 (59%)	7 (88%)	8 (44%)
AAV Diagnosis			n/a
○ GPA	6 (35%)	5 (63%)	
○ MPA	10 (59%)	0 (0%)	
○ EGPA	1 (9%)	3 (37%)	
Organ Involvement			n/a
Constitutional	8 (47%)	3 (37%)	
Renal	11 (64%)	5 (63%)	
Chest	7 (41%)	8 (100%)	
ENT	5 (29%)	7 (88%)	
Nervous	5 (29%)	3 (37%)	
Cutaneous	3 (17%)	1 (12%)	
Mucous membranes / eyes	1 (9%)	0 (0%)	
Disease duration, median (range), years	1.58 (0.01 – 8.5)	6.5 (1-29)	n/a
BVAS Score, median (range)	7 (2-26)	0	n/a
ANCA status at time of sampling			n/a
MPO-ANCA	9 (53%)	1 (12%)	
PR3-ANCA	6 (35%)	4 (50%)	
ANCA-negative	2 (12%)	3 (37%)	
Serum Creatinine (µM/L)	99 (57-582)	80 (69-122)	n/a
Raised CRP > 4 mg/L	9 (53%)	4 (50%)	n/a
eGFR < 60 mL/min	9 (53%)	1 (12%)	n/a
Therapy			n/a
Prednisolone	17 (100%)	6 (75%)	
Cyclophosphamide	8 (47%)	0 (0%)	
Rituximab	5 (29%)	1 (12%)	
Methotrexate	5 (29%)	4 (50%)	
Azathioprine	0 (0%)	2 (25%)	
Mycophenolate mofetil	2 (12%)	2 (25%)	

Abbreviations; AAV – ANCA-associated Vasculitis; ANCA – Anti-neutrophil Cytoplasmic Antibodies; BVAS – Birmingham Vasculitis Score; CRP – C-reactive Protein; EGPA – Eosinophilic Granulomatosis with Polyangiitis; ENT - Ear, Nose, Throat; eGFR - estimated Glomerular Filtration Rate; GPA – Granulomatosis with Polyangiitis; MPA – Microscopic Polyangiitis; MPO – Myeloperoxidase; PR3 - Proteinase 3

Manuscript Figures

June 2019



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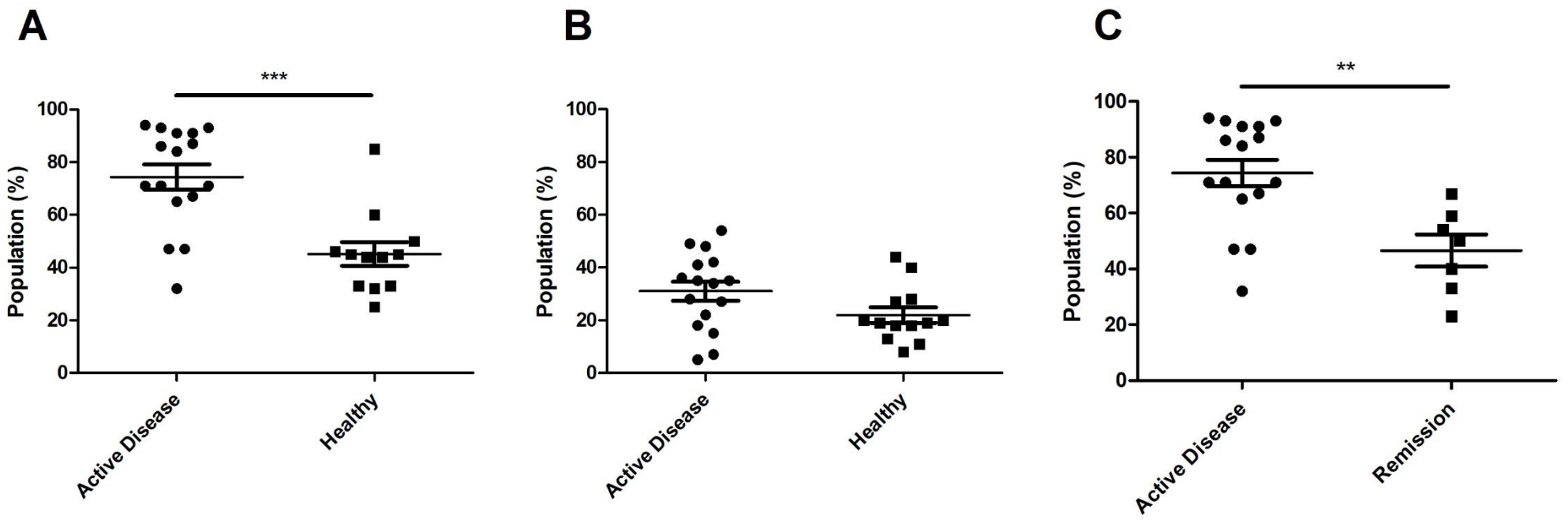

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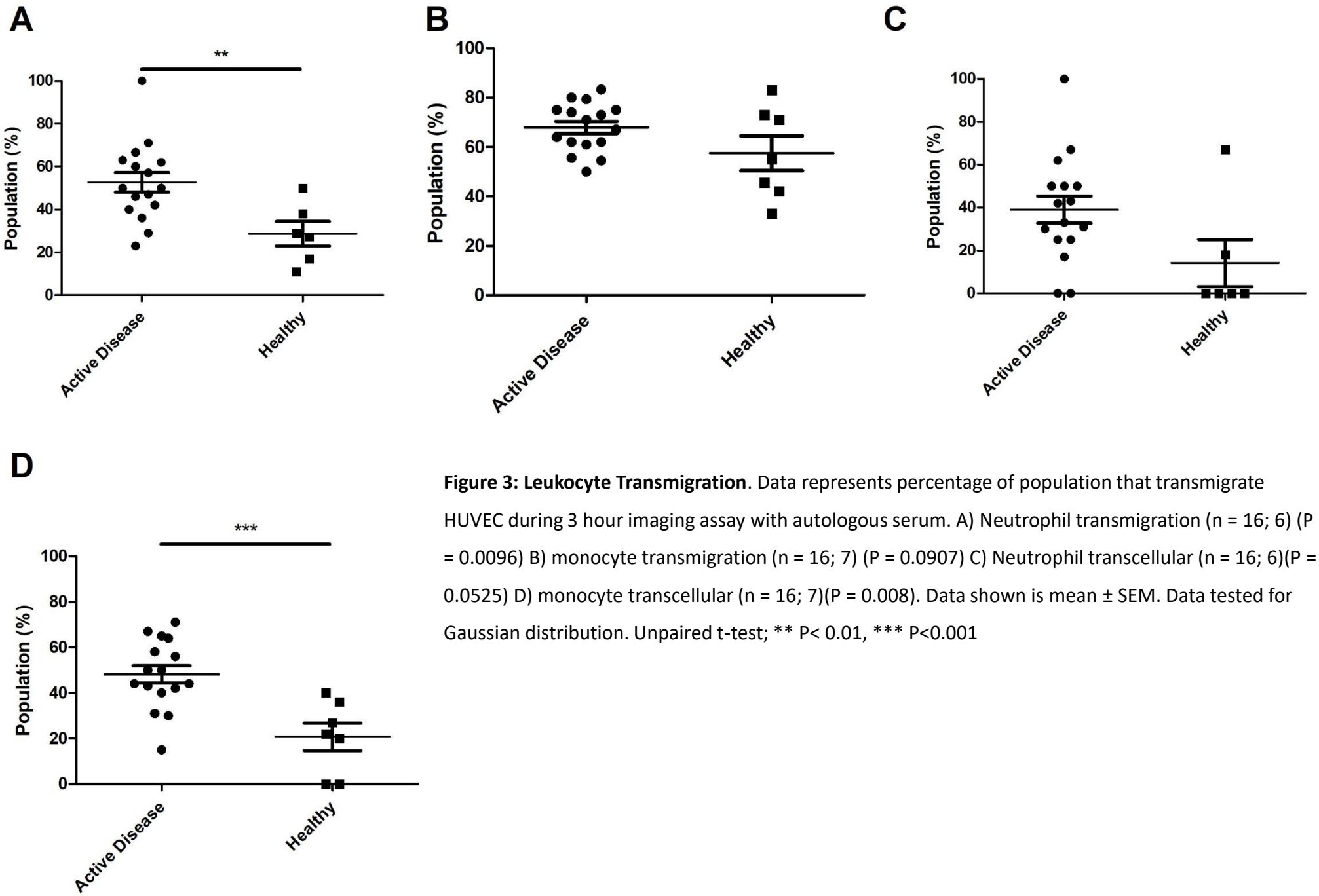

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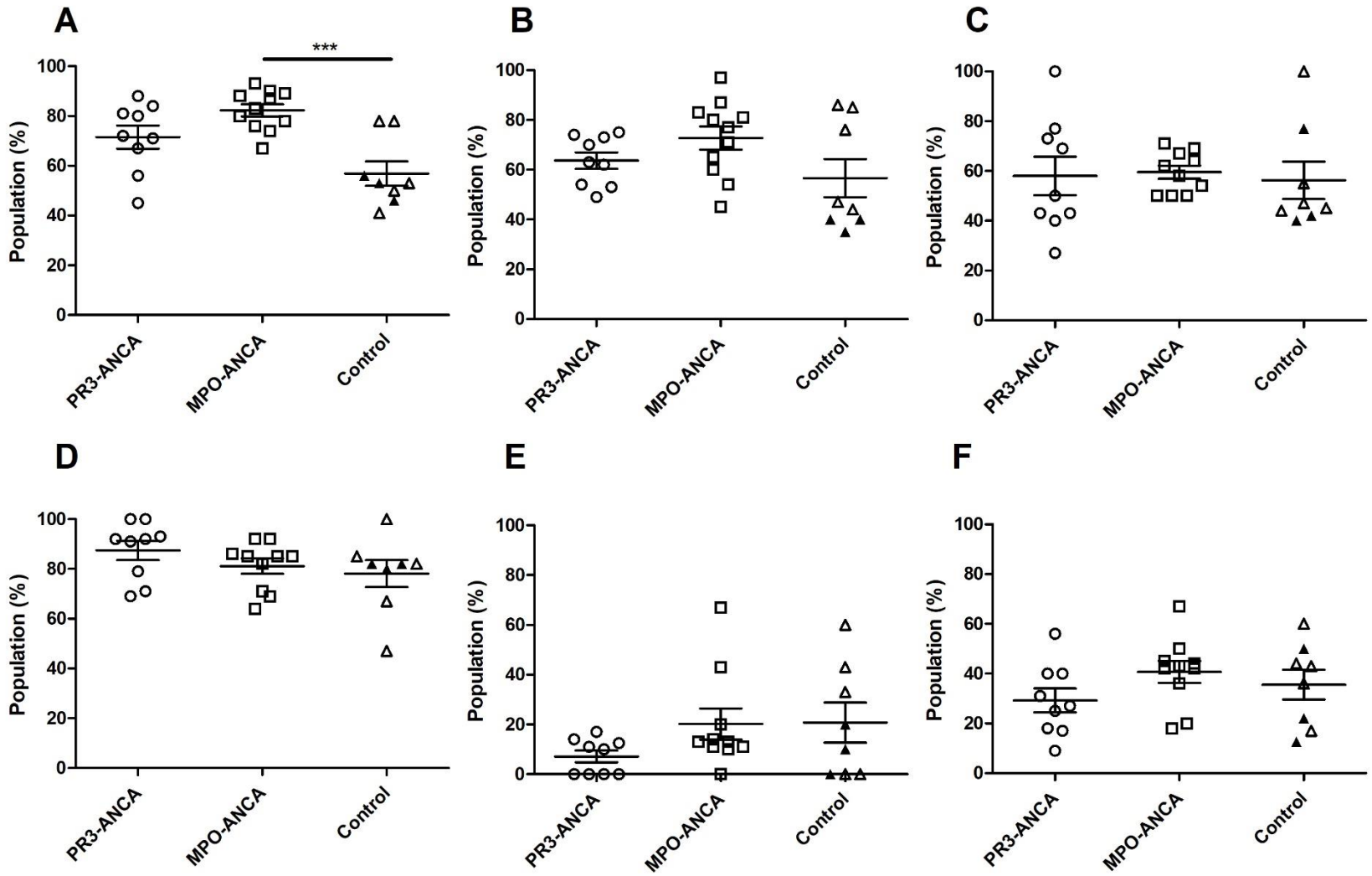

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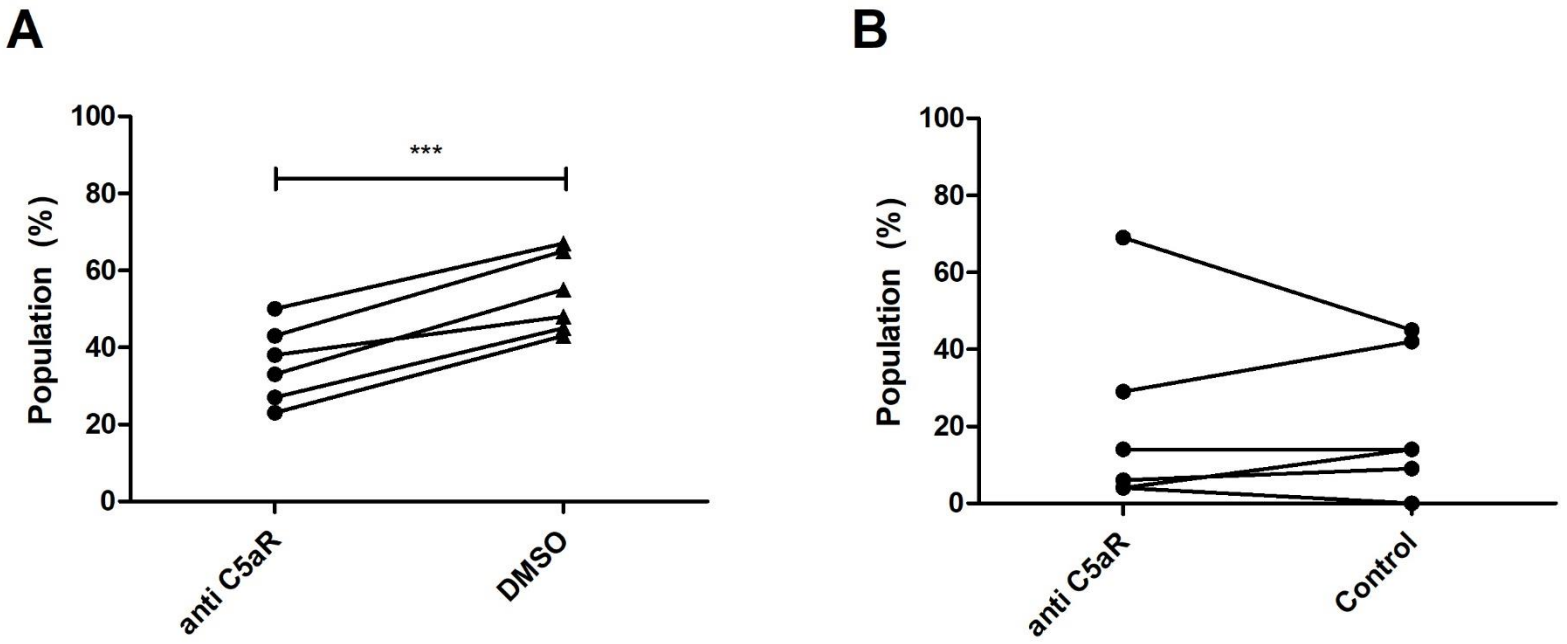

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