### 1 Multiplex immunohistochemistry differences between Q fever and atherosclerotic abdominal

### 2 aortic aneurysms indicate immune suppression

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### 1 Abstract

2	Background: Chronic Q fever is a zoonosis caused by the bacterium Coxiella burnetii which can
3	manifest as infection of an abdominal aortic aneurysm (AAA). Antibiotic therapy often fails, resulting
4	in severe morbidity and high mortality. Whereas previous studies have focused on inflammatory
5	processes in blood, the aim of this study was to investigate local inflammation in aortic tissue.
6	Methods: Multiplex immunohistochemistry was used to investigate local inflammation in Q fever
7	AAAs compared to atherosclerotic AAAs in aorta tissue specimen. Two six-plex panels were used to
8	study both the innate and adaptive immune system.
9	Results: Q fever AAAs and atherosclerotic AAAs contained similar numbers of CD68 <sup>+</sup> macrophages
10	and CD3 <sup>+</sup> T cells. However, in Q fever AAAs the number of CD68 <sup>+</sup> CD206 <sup>+</sup> M2 macrophages was
11	increased, while expression of GM-CSF was decreased compared to atherosclerotic AAAs.
12	Furthermore, Q fever AAAs showed an increase in both the number of CD8 $^{\scriptscriptstyle +}$ cytotoxic T cells and
13	CD3 <sup>+</sup> FoxP3 <sup>+</sup> regulatory T cells. Lastly, Q fever AAAs did not contain any well-defined granulomas.
14	Conclusions: These findings demonstrate that despite the presence of pro-inflammatory effector
15	cells, there is an immune suppressive micro environment in Q fever AAA resulting in persistent local
16	infection with <i>C. burnetii</i> .
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### 1 Introduction

2	Q fever is a zoonosis caused by the Gram-negative intracellular bacterium Coxiella burnetii (C.
3	burnetii), with natural reservoirs in a wide range of wild and domestic animals. In infected animals
4	(such as goats) milk, placenta and birth fluids, can contain this microorganism, which may cause
5	human infections via inhalation. Acute Q fever can present as pneumonia, hepatitis, and isolated
6	fever, yet 60% of cases are asymptomatic. (1) Progression to chronic Q fever occurs in approximately
7	5% of infected individuals (2); people at risk are older, suffer from renal insufficiency or aneurysm, or
8	previously underwent valvular or vascular prosthesis surgery. (3) Chronic Q fever manifests as
9	endocarditis or vascular Q fever, i.e., infection of an abdominal aortic aneurysm (AAA) or vascular
10	prosthesis. (4-6)

11

12	Vascular Q fever has severe clinical consequences. In a population of proven and probable vascular Q
13	fever patients according to the Dutch consensus guideline, a Dutch cohort study has described that
14	complications had occurred in 61% of the cases. Of these complications, acute aneurysms (i.e.
15	rupture, dissection, endoleak or symptomatic aneurysms) were most prevalent (35%), followed by
16	abscesses (22%), and fistula (14%). Moreover, 25% of patients had a definitely or probably chronic Q
17	fever related cause of death. (6) In addition, serological screening of 770 patients with aorto-iliac
18	disease, e.g., aneurysms or previous vascular reconstructions, demonstrated that 16.9% was
19	seropositive for Q fever, of which 30.8% suffered from chronic Q fever. In this group, aneurysm-
20	related acute complications were more common than in aneurysm patients without Q fever. (7)

21

To elucidate the pathology underlying chronic Q fever, previous studies have mainly focussed on
immune responses in peripheral blood. Blood mononuclear cells of patients with chronic Q fever,
when exposed to *C. burnetii* in vitro, produce high amounts of Interferon-gamma (IFNg), the

1	proinflammatory cytokine considered crucial for killing of the pathogen. (8, 9) In the infected tissues,
2	C. burnetii resides and replicates in monocytes and macrophages. (10) In vascular Q fever, it is
3	assumed that <i>C. burnetii</i> survives in resident macrophages in the vascular wall. (11, 12) In such
4	patients, there is an apparent inability to effectively eradicate C. burnetii, despite the
5	aforementioned IFNg response. In general, a pro-inflammatory response with granuloma formation
6	and intracellular killing or control of the bacterium by activated 'M1' monocytes/macrophages is
7	required to contain intracellular infections like Q fever. Surprisingly, the apparent inability of chronic
8	Q fever patients to kill C. burnetii has only been investigated in studies in vitro which showed
9	suggestive roles for polarization to tolerogenic M2 macrophages and increased numbers of
10	circulating regulatory T cells. (13, 14)
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11 12	It is still unclear how <i>C. burnetii</i> survives and locally escapes the immune system in vascular Q fever.
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12 13 14 15 16	To address this, we have investigated the local immune response in <i>C. burnetii</i> -infected AAAs (Q fever AAA), classical atherosclerotic abdominal aortic aneurysms (AAA), acutely infected AAA, and control aorta tissue, using multiplex immunohistochemistry (mIHC). We investigated both the adaptive and innate immune system. We show that in vascular Q fever numerous immune

### 1 Results

2

### 3 Baseline characteristics

- 4 This study includes ten Q fever AAAs (with C. burnetii PCR positive on aortic tissue), 12 classical
- 5 atherosclerotic AAAs, two acutely infected AAAs (with positive cultures of Streptococcus species),
- 6 and five normal abdominal aorta tissues. Table 1 presents baseline characteristics of the cohort,
- 7 showing that the majority of patients in all groups were males of older age (median 71 years old),
- 8 and cardiovascular risk factors were common, including hypertension, diabetes mellitus,
- 9 hypercholesterolemia, and smoking. Additionally, the aortic diameter was similar amongst the
- 10 groups.
- 11

Immune cell activation in Q fever, atherosclerotic, and acutely infected AAAs compared to normal
 aortas

14 Our mIHC technique reveals activation of both the innate and the adaptive immune system in Q 15 fever, atherosclerotic, and acutely infected AAAs compared to normal abdominal aortas (Figure 1). In 16 contrast to normal abdominal aortas, both Q fever AAAs and atherosclerotic AAAs showed 17 impressive lymphocyte accumulation and proliferation with very large tertiary lymphoid structures 18 (TLS) present in the adventitial layer (Figure 1F and 1J). Importantly, well-defined granulomas were 19 neither observed in any of the Q fever AAAs, nor in the other groups. In Q fever AAA, elevated 20 numbers of CD3<sup>+</sup> T cells (P=0.010) and CD20<sup>+</sup> B cells (P=0.012) were observed compared to normal 21 aortas. Atherosclerotic AAAs revealed increased numbers of CD3<sup>+</sup> T cells (P=0.005), CD20<sup>+</sup> B cells 22 (P=0.003), and CD15<sup>+</sup> neutrophils (P=0.023) compared to control. There were no significant 23 differences in the numbers of cells between Q fever AAA and atherosclerotic AAA. As expected, 24 acutely infected AAAs showed an increase in neutrophils compared to normal aortas (P=0.026). 25 Numbers of CD1c<sup>+</sup> classical dendritic cell type 2 (cDC2) and CD68<sup>+</sup> macrophages were similar among 26 all groups.

The principal component analysis (PCA) demonstrated when using these markers, a clear distinct population with normal aorta samples is formed, whilst the atherosclerotic and Q fever population completely overlapped (Figure 2A). Therefore, we aimed to investigate which markers differ between these two groups. If we add cell subset markers for macrophages and T cells, this overlap has completely disappeared, indicating these subset markers differentiate these groups (Figure 2B). Below, we will elucidate the differences in cell subsets between atherosclerotic AAA and Q fever AAA.

8

### 9 Q fever AAAs show a shift towards M2 macrophages

10 To investigate whether there are differences in innate immune system activation between 11 atherosclerotic AAA and Q fever AAA, we used mIHC for description of macrophage subset 12 populations based on CD68<sup>+</sup>CD206<sup>-</sup> (M1 macrophages) and CD68<sup>+</sup>CD206<sup>+</sup> (M2 macrophages) and for 13 expression of matrix metalloproteinase-9 (MMP9) and Granulocyte Macrophage Colony Stimulating 14 Factor (GM-CSF). As demonstrated in Figure 3, CD206 expression colocalized with CD68 in M2 15 macrophages. We found that in Q fever AAAs, the number of CD206<sup>+</sup> M2 macrophages was higher than in atherosclerotic aortas (P=0.005)(Figure 3J). These aortas also revealed lower levels of the pro-16 17 inflammatory cytokine GM-CSF when corrected for the percentage of macrophages and M2 18 macrophages (P=0.033 and P=0.007, respectively) (Figure 4). On the other hand, atherosclerotic 19 AAAs showed a larger amount of the more pro-inflammatory CD206<sup>-</sup> M1 subset compared to Q fever 20 AAAs (P=0.005)(Figure 3J), combined with a higher expression of GM-CSF per macrophage 21 (P=0.033)(Figure 4E). Additionally, we observed a larger MMP9<sup>+</sup> proportion of macrophages in 22 atherosclerotic AAAs than in Q fever AAAs (P=0.04). These findings are compatible with extensive 23 chronic inflammation but an immune-suppressed environment in Q fever AAAs in contrast to 24 atherosclerotic AAAs.

25

### 26 Increased cytotoxic T cells and regulatory T cells in Q fever AAAs

1 To study the involvement of the adaptive immune system in both groups, tissues were stained with 2 antibodies against CD8 for cytotoxic T cells, FoxP3 for regulatory T cells, and CD45RO for memory T 3 cells. Helper T cells were defined as CD3<sup>+</sup> T cells without CD8 expression. 4 Whereas the number of CD3<sup>+</sup> T cells is equal among vascular Q fever and atherosclerotic AAA 5 samples, samples from patients with vascular Q fever exhibited larger numbers of cytotoxic T cells 6 (P=0.000), coinciding with a decrease of helper T cells (P=0.000) and memory T cells (P=0.023) (Figure 7 5G). Infiltrates were formed, as depicted by the strong correlation between T- and B-cells 8 (correlation coefficient 0.6 [CI 0.404-0.788], P=0.000). 9 When correcting numbers of T cell subsets for infiltrate area, we found an increase in the number of cytotoxic T cells in Q fever AAA compared to atherosclerotic AAA (P=0.013)(Figure 5H). If 10 11 the number of T cell subsets is calculated per mm<sup>2</sup> tissue (defined as entire sample area minus 12 infiltrate area), the numbers of both cytotoxic T cells and regulatory T cells are increased in Q fever 13 AAA (P=0.043 and P=0.036 respectively)(Figure 5I). 14 15 Ruptured and non-ruptured aneurysms exhibit similar immune responses 16 Strikingly, only Q fever AAAs ruptured while the median diameter (60 mm) did not differ from the 17 diameter in the atherosclerotic AAA group (57 mm)(P=0.887). To investigate whether these patients 18 could be included in our analyses, we tested for differences between ruptured (N=3) and non-19 ruptured (N=21) aneurysms. Although signs of acute inflammation could be expected, no differences 20 were found in the number of T cells (P=0.206), B cells (P=0.407), cDC2 (P=0.150), neutrophils

21 (P=0.275), and macrophages (P=0.176). Also, when testing for cell subsets, ruptured and non-

22 ruptured aneurysms exhibited similar numbers of M1/M2 macrophages (P=0.206), cytotoxic T cells

- 23 (P=0.329), regulatory T cells (P=0.206), and memory T cells (P=0.176). The similarity of the results
- 24 between both groups may be explained by the sampling method; all samples were taken from the
- 25 ventral side of the aneurysm and the rupture side was unknown or not registered at the time.

1

### 2 Q fever aortas reveal extensive fibrosis

3	All above mentioned features are signs of chronic inflammation and long-existing disease. This was
4	supported by HE- and Elastin Van Gieson (EVG) stainings, which demonstrated destruction of elastin
5	fibers and fibrosis. Both atherosclerotic AAAs and Q fever AAAs exhibited extensive atherosclerotic
6	plaque formation. However, there were large differences in vessel architecture as demonstrated in
7	Figure 6. Earlier studies have extensively described that aortic aneurysms show fragmentation of
8	elastin fibers indicating media degeneration. (15, 16) In our series, the amount of elastin fibers was
9	even more decreased in many Q fever AAAs than in atherosclerotic AAAs with a similar diameter
10	(marked with black arrows). In addition, the tunica adventitia showed extensive fibrosis in Q fever
11	AAAs (marked with asterisks in Figure 6I and 6L). These changes indicate the (more pronounced)
12	disrupted architecture in Q fever AAAs, which can attribute to ongoing inflammation.

### 1 Discussion

2	We are the first to introduce mIHC in vascular Q fever to study ongoing local inflammation. This
3	sophisticated mIHC method enabled us to quantify immune cells in large sections of tissue which
4	minimized sampling bias. First, we showed that granulomas are absent in Q fever AAAs. Second,
5	atherosclerotic and Q fever AAAs were similar when comparing numbers of immune cells. However,
6	there were striking differences in the composition of macrophage- and T cell-phenotypes between
7	AAAs and Q fever AAAs, leading to new insights into the pathogenesis of vascular Q fever and its
8	complications and possibly with therapeutic consequences.

9

10 Our first observation, the absence of well-formed granuloma formation in our cohort of Q fever AAAs 11 is an important one, since it suggests that the local immune landscape lacks an adequate pro-12 inflammatory response. In our series, we could not find any well-formed granuloma similar to how they are described in acute Q fever. In acute Q fever, so called doughnut granulomas are reported: 13 14 granulomas with a central clear space and a fibrin ring within or at its periphery (1, 17), for example 15 in liver biopsies in case of hepatitis. (18) Here, granuloma is a feature of active defense against the 16 pathogen. This contrasts to chronic Q fever, where granulomas have not been described before. (17, 17 19) In particular, Lepidi described that resected valve specimens of patients with Q fever endocarditis 18 lacked well-formed granulomas. (12) In vascular Q fever, granulomatous responses consisting of 19 histiocytes surrounding necrotic areas have been reported in Q fever AAAs, however well-formed 20 granulomas were not found. (20) Thus, we would interpret the absence of organized granulomas as 21 the first clue for an immune-suppressed environment in AAA of Q fever patients that allows 22 persisting infection after the acute phase.

23

Secondly, our results demonstrate some similarities between Q fever AAAs and AAAs. Percentages of
CD3<sup>+</sup> T cells, CD20<sup>+</sup> B cells, CD1c<sup>+</sup> cDC2, CD15<sup>+</sup> neutrophils, and CD68<sup>+</sup> macrophages are similar
between the groups with atherosclerotic AAA and Q fever AAA. This finding is supported by the PCA,
which shows overlapping populations of atherosclerotic and Q fever AAAs when entering these
inflammatory cell markers. This does not come as a surprise since it is well established that vascular
Q fever develops in preexisting atherosclerotic aneurysms. (5, 7, 20-22)

7

8 Despite the similarities, we discovered that atherosclerotic and Q fever AAAs do have important 9 differences, which emerge when investigating macrophage and T cell subset markers. Macrophages 10 in Q fever AAAs were found to be polarized into the less inflammatory M2 phenotype, which is 11 'tolerogenic' and poorly microbicidal, in contrast to the M1 phenotype that possesses a machinery 12 that can clear an infection. Interestingly, in the AAAs we found less M2 polarization. This would 13 either indicate that macrophages polarize towards M2 in response to C. burnetii infection, or that the 14 presence of M2 polarization is a prerequisite for *C. burnetii* persistence. Previous studies have 15 demonstrated that C. burnetii inhabits and proliferates in monocytes and macrophages, and more 16 specifically, in resident vascular wall macrophages in case of vascular Q fever. (10, 12) It has been 17 shown by Benoit et al. that C. burnetii stimulates an atypical M2 activation program in monocyte-18 derived macrophages in vitro. (14) M2 polarization of macrophages was also observed in C. burnetii 19 infected transgenic mice constitutively expressing IL-10 in macrophage lineage, a mouse model for 20 chronic Q fever pathogenesis. (23) Spleens and livers of these mice showed increased expression of 21 arginase-1 and mannose receptor (CD206) and decreased expression of iNOS, IL-12 and IL-23 in 22 bone-marrow derived macrophages after infection with C. burnettii compared to C. burnetii-infected 23 wild type mice. These previous findings suggested that chronic Q fever is associated with M2 24 polarization of macrophages, but direct evidence in chronic Q fever patients was lacking. Our findings

establish that outgrowth and persistence of *C. burnetii* in AAAs is associated with the predominance
 of M2 macrophages.

3

4 There are several possible explanations for the lack of macrophage activation. First, our results 5 demonstrate decreased expression of GM-CSF in Q fever AAAs compared to AAAs. GM-CSF is a pro-6 inflammatory cytokine that activates granulocytes and macrophages. (24) Its decreased expression in 7 Q fever AAAs may contribute to the immune suppressive environment in Q fever AAA. The role of 8 GM-CSF in the context of aneurysm formation has been investigated previously. (25) Strikingly, Son 9 et al described increased occurrence of aortic dissection and intramural hematoma in wild type mice 10 subjected to aortic inflammation (CaCl2 + Ang II administration) when also receiving GM-CSF. Only 11 administrating GM-CSF, without the prerequisite of aortic inflammation, did not result in aortic 12 dissection or intramural hematoma. Its potential clinical relevance was confirmed in human blood: 13 GM-CSF serum levels of patients suffering from acute dissection were higher than controls with 14 coronary artery disease, aortic aneurysms of healthy volunteers. (25) Additionally, in our cohort we 15 found that Q fever AAAs rupture at smaller diameter compared to atherosclerotic AAA. This finding, 16 combined with the GM-CSF paradox, suggests that the development of Q fever AAAs and 17 atherosclerotic AAAs follow different pathways, however strictly hypothetically.

18

Second, a key cytokine in activation is IFNg, a T-helper (Th)-1 cytokine that activates macrophages and makes them more microbicidal. Previous studies from our group have demonstrated that peripheral blood mononuclear cells from patients with chronic Q fever exhibit an abundant production of IFNg when exposed to *C. burnetii* antigens. (8, 9) These findings were enigmatic since there is an apparent inability of the patient's immune system to kill *C. burnetii* at the infected sites. The current findings would be compatible with a downregulated IFNg response at the infected site.

1

3First, the number of cytotoxic T cells was increased in both infiltrate and surrounding tissue of Q4fever AAA compared to AAA. Although the numbers of cytotoxic T cells were high, their function5might be compromised, resulting in defective elimination of <i>C. burnetii</i> . The increased numbers of6regulatory T cells we found in Q fever AAAs may play a role here. An increased number of circulating7regulatory T cells has also been shown by Layez et al in Q fever endocarditis patients and in acute Q8fever patients. (13) Regulatory T cells can inhibit cytotoxic T cells directly or indirectly (26), with a9possible role for IL-10 produced by this T cell subset. An important role of IL-10 in chronic10development of Q fever has been postulated based on converging evidence from a series of <i>in vitro</i> 11studies. IL-10 production by peripheral blood mononuclear cells from patients with Q fever12endocarditis and Q fever with valvulopathy who were at risk for developing chronic Q fever was high,13compared to control individuals. (27, 28) Moreover, IL-10 specifically increases <i>C. burnetii</i> replication14in naive monocytes (29) possibly by downregulating IFNg. Finally, low IL-10 production in monocytes15from patients with acute Q fever was associated with <i>C. burnetii</i> elimination, whereas <i>C. burnetii</i> 1810. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in19bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the10macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed<	2	In addition to differences in macrophage subsets, differences in T cell subsets were also observed.
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<ul> <li>development of Q fever has been postulated based on converging evidence from a series of <i>in vitro</i></li> <li>studies. IL-10 production by peripheral blood mononuclear cells from patients with Q fever</li> <li>endocarditis and Q fever with valvulopathy who were at risk for developing chronic Q fever was high,</li> <li>compared to control individuals. (27, 28) Moreover, IL-10 specifically increases <i>C. burnetii</i> replication</li> <li>in naive monocytes (29) possibly by downregulating IFNg. Finally, low IL-10 production in monocytes</li> <li>from patients with acute Q fever was associated with <i>C. burnetii</i> elimination, whereas <i>C. burnetii</i></li> <li>replicated in monocytes from patients with chronic Q fever and high IL-10 production. The</li> <li>microbicidal activity of monocytes from patients with chronic Q fever was restored by neutralizing IL-</li> <li>10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in</li> <li>bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the</li> <li>macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed</li> </ul>	8	fever patients. (13) Regulatory T cells can inhibit cytotoxic T cells directly or indirectly (26), with a
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<ul> <li>compared to control individuals. (27, 28) Moreover, IL-10 specifically increases <i>C. burnetii</i> replication</li> <li>in naive monocytes (29) possibly by downregulating IFNg. Finally, low IL-10 production in monocytes</li> <li>from patients with acute Q fever was associated with <i>C. burnetii</i> elimination, whereas <i>C. burnetii</i></li> <li>replicated in monocytes from patients with chronic Q fever and high IL-10 production. The</li> <li>microbicidal activity of monocytes from patients with chronic Q fever was restored by neutralizing IL-</li> <li>10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in</li> <li>bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the</li> <li>macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed</li> </ul>	11	studies. IL-10 production by peripheral blood mononuclear cells from patients with Q fever
<ul> <li>in naive monocytes (29) possibly by downregulating IFNg. Finally, low IL-10 production in monocytes</li> <li>from patients with acute Q fever was associated with <i>C. burnetii</i> elimination, whereas <i>C. burnetii</i></li> <li>replicated in monocytes from patients with chronic Q fever and high IL-10 production. The</li> <li>microbicidal activity of monocytes from patients with chronic Q fever was restored by neutralizing IL-</li> <li>10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in</li> <li>bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the</li> <li>macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed</li> </ul>	12	endocarditis and Q fever with valvulopathy who were at risk for developing chronic Q fever was high,
from patients with acute Q fever was associated with <i>C. burnetii</i> elimination, whereas <i>C. burnetii</i> replicated in monocytes from patients with chronic Q fever and high IL-10 production. The microbicidal activity of monocytes from patients with chronic Q fever was restored by neutralizing IL- 10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed	13	compared to control individuals. (27, 28) Moreover, IL-10 specifically increases C. burnetii replication
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<ul> <li>microbicidal activity of monocytes from patients with chronic Q fever was restored by neutralizing IL-</li> <li>10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in</li> <li>bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the</li> <li>macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed</li> </ul>	15	from patients with acute Q fever was associated with C. burnetii elimination, whereas C. burnetii
<ul> <li>10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in</li> <li>bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the</li> <li>macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed</li> </ul>	16	replicated in monocytes from patients with chronic Q fever and high IL-10 production. The
<ul> <li>bacterial persistence. <i>C. burnetii</i> infection is persistent in mice that overexpress IL-10 in the</li> <li>macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed</li> </ul>	17	microbicidal activity of monocytes from patients with chronic Q fever was restored by neutralizing IL-
20 macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed	18	10. (30) The murine model of chronic Q fever mentioned above, also confirmed a key role for IL-10 in
	19	bacterial persistence. C. burnetii infection is persistent in mice that overexpress IL-10 in the
21 environment.	20	macrophage compartment. (23) Thus, IL-10 could play a crucial role in this immune-suppressed
	21	environment.

22

The last major difference between Q fever AAA and atherosclerotic AAA is the extent of damage to
the vascular wall architecture in Q fever AAAs. This is demonstrated by extensive loss of elastin fibers
and increase of fibrosis present in the vascular wall. Fragmentation of elastin fibers has been

described for AAAs previously (16), however we found the loss of elastin fibers more evident in the
lesions from Q fever AAAs than atherosclerotic AAAs. Fibrosis is characterized by replacement of
normal tissue by excessive connective tissue, and usually follows chronic inflammation. This may be
the effect of persistent presence of growth factors, proteolytic enzymes, angiogenic factors, and
profibrotic cytokines. (31, 32) Previously, fibrosis was also observed in chronic Q fever endocarditis in
humans and cows. (12, 33-35) This indicates that our Q fever AAA cohort suffered from more
destructive disease than our AAA cohort.

8

9 These novel insights could lead to new clues for novel treatments and thus developments for clinical 10 care. Currently, Q fever AAA still leads to significant morbidity and mortality rates despite antibiotic 11 and surgical treatment. Epidemiological studies demonstrate the similar risk profile of Q fever and 12 non-Q fever infected AAAs, yet the risk of complications is higher in the Q fever infected group (7), 13 even up to 61% (6), and 25% of patients suffering from Q fever AAA had deceased with a 14 definitely/probably chronic Q fever related cause of death. (6) Here, we confirm that in vascular Q fever, the local immune response is skewed towards an immunotolerant state. Hypothetically, the 15 16 decreased expression of GM-CSF suggests a possible role for immunomodulating treatment, for 17 example with administration of recombinant GM-CSF. This is already approved for neutropenia due 18 to myelosuppression (36), and has been suggested for treatment for pulmonary tuberculosis. (37) 19 There might be a role for immunomodulating adjuvant therapies in patients with Q fever AAA in 20 whom treatment failure is observed with antibiotics alone.

21

Our study was the first to use mIHC in Q fever AAA and thereby to gain information about the
 number and proportion of immune cells, and simultaneously obtain spatial information. This
 powerful technique and the access to rare Q fever AAA tissue are strengths of this study. While other
 studies have tested for immune cell activation and recruitment in peripheral blood, we were able to

1 study the actual infected tissue. Interpreting our results in context of previous observations enables 2 us to increase our understanding of the pathophysiology of Q fever AAA. Still, several limitations 3 should be noted. First, our sample size is limited with only ten vascular Q fever samples. However, 4 this is still the largest study investigating local immune responses in Q fever AAA in humans. In 5 addition, in our quantification method we include entire slides up to 238 20x views per patient, 6 which minimizes the effects of the small sample size. Second, consistent with IHC studies in general, 7 we can only describe the immune cells we observe, without answering mechanistic questions. 8 Nevertheless, when interpreting our results in light of the current literature, we can reasonably 9 formulate hypotheses about the pathophysiology and test these in further research. 10 11 Taken together, this leads to the following model with a prominent role for immune suppression. 12 First, macrophages that harbor C. burnetii are not effectively killing the organisms, probably due to a 13 lack of activation by proinflammatory cytokines like GM-CSF and IFN-g in a microenvironment with 14 excess IL-10. Second, effector T cells that attempt to eliminate the intracellular bacterium residing in 15 monocytes and macrophages, are hindered by regulatory T cells that are prominent IL-10 producers. 16 Third, there is a lack of microbicidal M1 macrophages, instead macrophages are polarized into the 17 tolerogenic M2 phenotype, which leads to insufficient attack of the pathogen, enabling persistent

18 infection.

### 1 Methods and materials

Abdominal aorta tissue samples from patients with Q fever infected aneurysms and control groups
were investigated with a novel mIHC method to study the involvement of the innate and adaptive
immune system in vascular Q fever. The data underlying this article will be shared upon reasonable
request to the corresponding author.

6

### 7 Patient samples

8 Tissue samples were collected from four groups of patients in two Dutch hospitals: Jeroen Bosch 9 Hospitals in 's Hertogenbosch and Radboud university medical center in Nijmegen. The first group 10 consisted of patients diagnosed with C. burnetii infected AAA (Q fever AAA) according to the Dutch consensus guideline (38): all patients had an abdominal aneurysm (AAA) and IgG phase I was at least 11 12 1:1024 in combination with a positive PCR of aortic tissue. The second group consisted of patients 13 with atherosclerotic atherosclerotic AAA without clinical suspicion of Q fever. The third group 14 consisted of patients with an acutely infected AAA, with the same definition of AAA in combination 15 with positive cultures of Streptococcus pneumoniae and Streptococcus Agalactiae, respectively. In 16 these three groups AAA was defined as a CT proven abdominal aortic aneurysm with a diameter of at 17 least 3.0 cm. (39) All aneurysmatic tissue samples were either obtained from patients undergoing 18 elective surgical repair or emergency repair in case of aortic rupture. The fourth group consisted of 19 abdominal aorta samples from patients undergoing kidney explantation surgery for transplantation 20 purposes, with an aortic diameter smaller than 3.0 cm. The samples from Jeroen Bosch Hospital were 21 described in a previous study. (20)

The medical ethics committees of the institutions approved the study, in line with the principles
outlined in the Declaration of Helsinki (Radboudumc: 2017-3196; Jeroen Bosch Hospital:
2019.05.02.01).

1

### 2 Tissue Processing

3	During surgery, the ventral part of the abdominal aorta was removed. If necessary, adhering thrombi
4	were gently removed from the tissue before further processing. Directly after collection, samples
5	were fixed in buffered 4% formaldehyde for at least 24 hours and no longer than 72 hours. If large
6	amounts of calcification were present, samples were decalcified by storing them in EDTA solution for
7	another 24 hours. Subsequently, samples were carefully embedded in paraffin in an attempt to
8	include all aorta layers (formalin fixed and paraffin embedded (FFPE)). Of these tissues, full thickness
9	transverse sections of 4 $\mu m$ were mounted on silane coated glass slides (New Silane III, MUTO PURE
10	CHEMICALS, Japan).
11	
12	Multiplex Immunohistochemistry
13	Samples were stained with two mIHC panels, which enclosed the innate and adaptive immune
14	system (Table 2). Optimization and validation of mIHC panels were performed as described
15	previously. (40) Samples were stained with six consecutive tyramide signal amplification (TSA) stains
16	followed by antigen stripping after every staining. This resulted in the fluorophore remaining on the

17 target, thus enabling eight simultaneous colors on one slide (six markers, DAPI and

18 autofluorescence). Slides were stained automatically in a Leica Bond system (BOND-Rx Fully

19 Automated IHC and ISH, Leica Biosystems). After positioning in the machine, slides were

20 deparaffinized, rehydrated and washed with demi water. After this, samples underwent heat induced

21 antigen retrieval (HIER) in BOND Epitope Retrieval 2 (AR9640, Leica Biosystems) or BOND Epitope

22 Retrieval 1 (AR9961, Leica Biosystems) during 20 minutes for the adaptive and innate panel,

23 respectively. Then, protein blocking in Akoya Antibody Diluent/Block (Akoya biosciences, MA) took

24 place for 10 minutes, followed by incubation with the first primary antibody for 1 hour, subsequently

with the secondary antibody (Polymer HRP, Ms + Rb (Akoya biosciences, MA)) for 30 minutes and

1 finally with an Opal fluorophore ((Akoya biosciences, MA) dissolved 1:50 in 1 X Plus Amplification 2 Diluent (Akoya biosciences, MA) for 10 minutes. To facilitate multiplex staining with six markers, 3 samples were heated for 10 minutes which enabled antigen stripping. After this staining cycle, this 4 procedure was repeated for five different primary antibodies, the secondary antibody and 5 corresponding Opal fluorophores. Finally, DAPI was used as a nuclear counterstain and slides were 6 mounted with Fluoromount-G (0100-01; Southern Biotech, Birmingham, AL, USA). All incubations 7 steps were performed at room temperature. Please see Supplementary Table 2 for a more detailed 8 overview of the used reagents.

9

### 10 Imaging, multispectral unmixing and analysis

After staining, image acquisition and immune cell quantification were performed using an automated approach. First, the PerkinElmer Vectra (Vectra 3.0.3; PerkinElmer, MA) scanned whole slides at 4x magnification and 20x magnification, allowing precise cell segmentation incorporating the entire sample. The average 20x views per slide was 283 resulting in an average tissue area of 59.0 ± 32.6 mm<sup>2</sup>, and this high number substantially reduces the chance of sampling bias. Spectral libraries and inForm Advanced Image Analysis software (inForm 2.4.8; Akoya biosciences, MA) unmixed these multispectral images (Supplementary Figures 1A and 2A).

18 Subsequently, inForm Advance Imaging Analysis software was used for segmentation of tissue and 19 cells. For tissue segmentation, tissue slides were divided into tissue, infiltrate, thrombus, blood, and 20 background (Supplementary Figure 1C). This segmentation was based on DAPI, autofluorescence 21 and, if present, also CD20 and CD3. For single cell segmentation, cells were identified with DAPI and 22 autofluorescence, and, depending on the panel, with membrane markers CD20 and CD3 23 (Supplementary Figure 1B) or CD68, CD206 and CD15. Requiring DAPI for cell segmentation ensured 24 the exclusion of artifact staining, in which DAPI is absent. The output of the software was 20x 25 magnification images and cell data (localization, tissue, phenotype, and marker) per slide. Images

1	were combined into single flow cytometry standard (fcs) files, allowing analysis in FlowJo (FlowJo
2	10.0.7, Becton Dickinson, NJ). In FlowJo, only cells in tissue and infiltrate were analyzed and gates
3	were drawn as shown in Supplementary Figure 1E for the adaptive panel and Supplementary Figure
4	2B for the innate panel by two observers with excellent interobserver correlation (Supplementary
5	Figure 1D and 2D).
6	These clear distinct positive cell populations were not found for CD45RO and MMP9 as their
7	expression is gradual. (41) For that matter, the gates for these markers were drawn in negative
8	populations, namely non-T cells and non-neutrophils, respectively. Following this, these gates were
9	copied to populations that could express these markers. GM-CSF fcs files did not show distinct
10	positive and negative populations although they were visible at the microscopy images. Therefore,
11	inForm Advance Imaging Analysis software was used for automatically thresholding for GM-CSF per
12	sample, providing the number of GM-CSF positive pixels per sample (Supplementary Figure 2C).
13	
14	Histology
15	To study vessel wall architecture, tissue samples were stained with hematoxylin-eosin (HE) and
16	Elastin von Gieson (EVG).
17	
18	Statistical analysis
19	SPSS for Windows (IBM Corp, 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM
20	Corp) was used for statistical analysis. PRISM 8.0.2 (Graphpad, GSL Biotech LLC, CA) was used for
21	visualization of results. Continuous data were expressed as mean $\pm$ standard deviation (SD), or in

22 case of non-Gaussian distribution, as median (interquartile range) (IQR). Kruskal-Wallis test adjusted

23 with Bonferroni correction for multiple testing was used for testing continuous variables between

four groups. Binary variables were tested for differences using the Fisher exact test. Interobserver
variability was calculated with the intraclass correlation coefficient (ICC). Correlations between
continuous non-Gaussian distributed variables were studied with Kendall's tau because of low
numbers per group. P < 0.05 was considered statistically significant. Principal component analysis</li>
was performed in RStudio 1.2.5033 (RStudio, inc. Boston, MA) and R (R Foundation for Statistical
Computing, Vienna, Austria) using singular value decomposition and the tidyverse (42) and factoextra
packages. (43)

8

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15

16

- 17 Competing interests
- 18 None declared.

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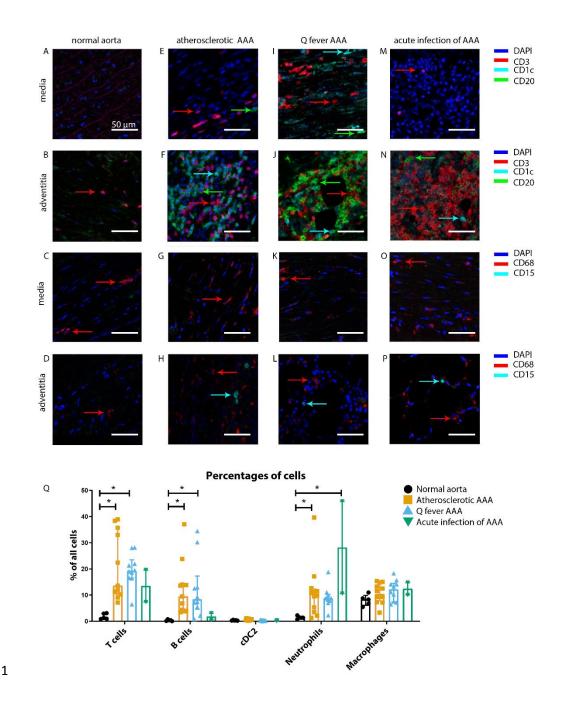
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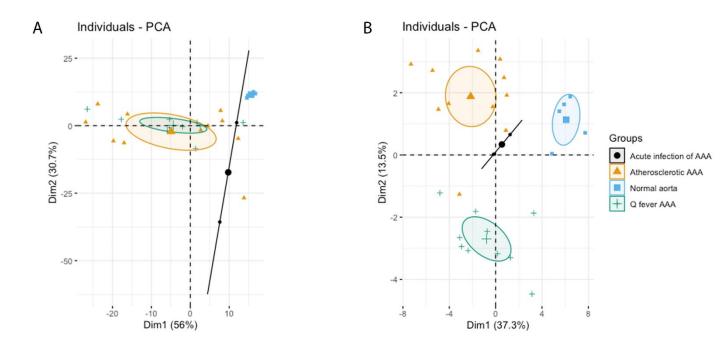
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### 1 Figures with corresponding legends



2 Figure 1: Immune cell activation in atherosclerotic, Q fever infected and acutely infected AAAs. All scale bars represent 50 3 μm. A-P: Adaptive (A, B, E, F, I, J, M, N) and innate (C, D, G, H, K, L, O, P) immune cells in a representative normal abdominal 4 aorta, atherosclerotic AAA, Q fever AAA and acutely infected AAA. Arrows with corresponding colors indicate presence of 5 immune cells with red for CD3<sup>+</sup> T cells, cyan for CD1c<sup>+</sup> cDC2, green for CD20<sup>+</sup> B cells in the adaptive panel (A, B, E, F, I, J, M, 6 N); and red for CD68<sup>+</sup> macrophages and cyan for CD15<sup>+</sup> neutrophils in the innate panel (C, D, G, H, K, L, O, P). Q: 7 quantification of percentages of different types of immune cells in the whole tissue sections, showing the increases in T and 8 B cells in atherosclerotic AAA and Q fever AAA compared to normal and increase in neutrophils in acute infection and 9 atherosclerotic AAA compared to normal. Note that there are no differences between atherosclerotic AAA and Q fever AAA.

10 \* Represents  $P \le 0.05$ .





2 Figure 2: A: Principal component analysis (PCA) including CD3, CD20, CD68, CD15, and CD1c. There is a clear distinct

3 population consisting of normal abdominal aortas. There are two data points for acute infection, resulting in a line.

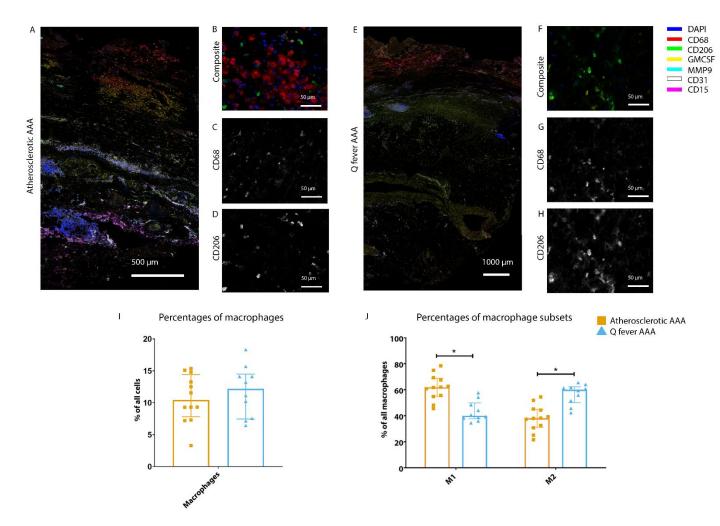
4 Intriguingly, atherosclerotic AAA and Q fever infected AAA are completely overlapping. This indicates that these populations

5 are similar when testing for these cell markers. B: PCA including all markers (CD68, CD15, MMP9, GMCSF, CD31, CD206,

6 CD3, CD1c, CD8, FoxP3, CD45RO, and CD20). Note the difference with figure 2A: here all groups form separate populations,

7 indicating that the newly added markers including subset markers describe the differences between atherosclerotic and Q

8 fever AAA. See Supplementary Table 1 for loadings of both PCAs.



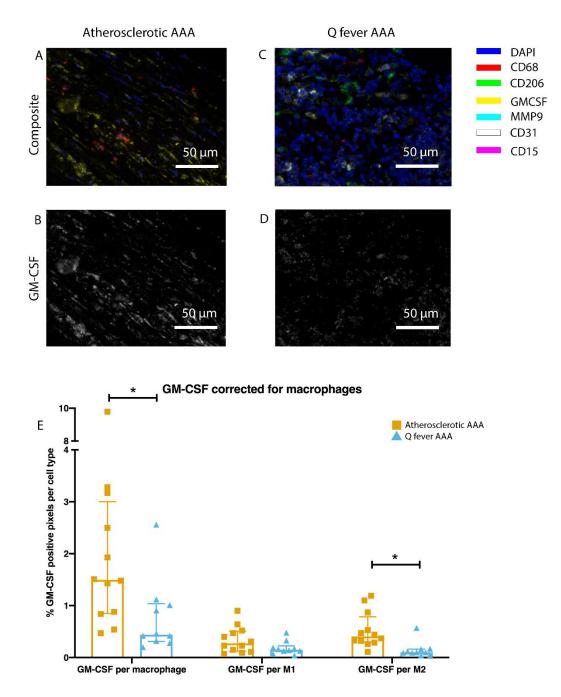
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Figure 3: Phenotype shift in macrophages in Q fever towards M2. A: Overview photo of atherosclerotic AAA, upper portion
 is intima layer, lower portion adventitia. B: Composite of CD68 and CD206 with majority CD68. C, D: Separated channels for
 CD68 and CD206 respectively. E: Overview of Q fever infected AAA, with the same orientation as A. F: Composite of CD68

5 and CD206, with mostly CD206+ cells which also express CD68, as supported by separated channels in G and H. I, J:

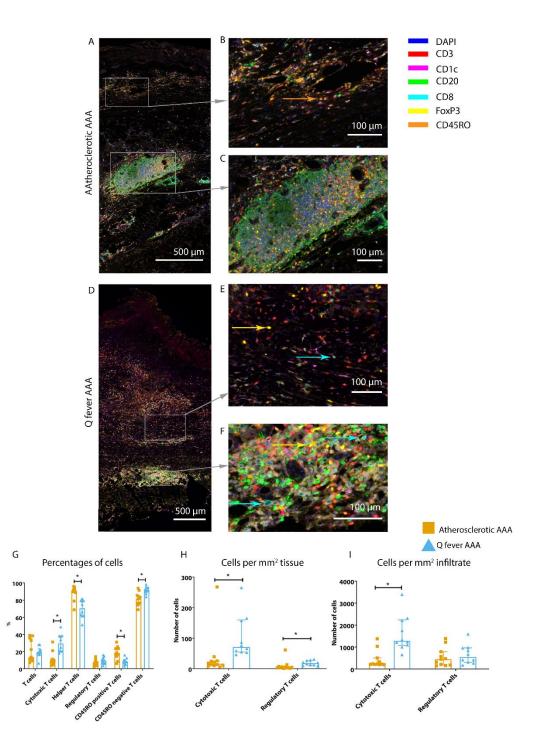
6 Quantification of percentages of macrophages in entire tissue sections (I) and of proportions of M1 and M2 macrophages in

7 these macrophages (J), showing the phenotype switch in Q fever AAAs towards M2. \* Represents  $P \le 0.05$ .



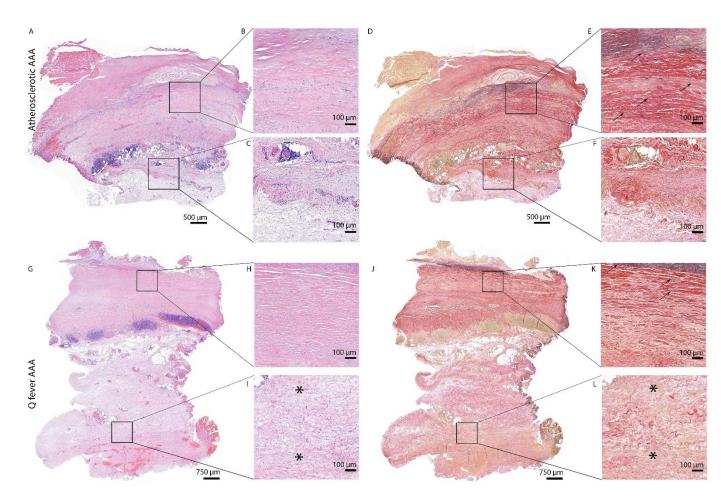
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Figure 4: Q fever infected AAAs express lower levels of GM-CSF. A-D: Representative composite image of atherosclerotic
 AAA (A) and Q fever AAA (C) and corresponding GM-CSF channels (B, D). E: The expressed levels of GM-CSF corrected for
 the number of macrophages and M2 macrophages are lower in Q fever infected AAAs, suggesting an immune suppressed
 environment.

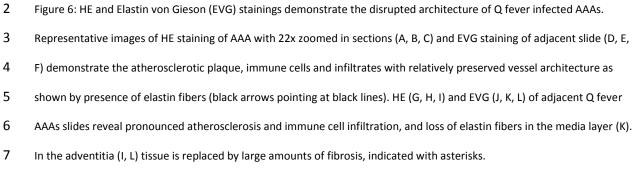


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Figure 5: Q fever AAAs exhibit both pro-inflammatory and anti-inflammatory T cell subsets. Arrows with corresponding
colors indicate presence of immune cells, with orange for memory T cells, yellow for T helper cells, and cyane for cytotoxic T
cells. A-F: Overview of atherosclerotic AAA (A) and Q fever AAA with zoomed photos of tissue (B, E) and tertiary lymphoid
structures (TLS)(C, F). In both A and B the upper side of the photo is the intima layer. Note all the FoxP3+ (yellow) cells in Q
fever infected tissue. G: Percentage of T cells of all cells and T cells subsets out of T cells; G, H, I: Quantification shows a shift
in cytotoxic / helper T cell ratio and decrease in memory T cells in Q fever AAAs. Q fever AAAs show increased numbers of
cytotoxic and regulatory T cells, indicating both immune activation and suppression.







### 1 Tables

Characteristic	Normal (N=5)	Missing data	Atherosclerotic	Missing data	Q fever AAA	Missing	Infectious	Missing data	Significance
		normal	AAA (N=12)	atherosclerotic	(N=10)	data Q fever	AAA (N=2)	infectious	
Male	3 (60%)	2	10 (83.3%)	0	9 (90.0%)	1	1 (50.0%)	1	0.482
Age	65 (54-68)	0	72 (66-78)	0	71 (64-77)	0	71 (71-71)	1	0.318
Length	1.80 (1.69-1.83)	0	1.77 (1.69- 1.80)	0	1.78 (1.70-)	8	1.76-1.76- 1.76)	1	0.923
Weight	80.0 (77.5- 102.5)	0	82.6 (75.5- 92.6)	0	88.5 (79-)	8	94.8 (94.8- 94.8)	1	0.637
BMI	27.0 (22.1-34.7)	0	26.8 (24.0- 29.4)	0	27.8 (27.3-)	8	30.6 (30.6- 30.6)	1	0.651
Hypertension	3 (60%)	0	8 (66.7%)	0	6 (60.0%)	2	1 (50.0%)	1	1.000
Hypercholestero	lemia	5	9 (75.0%)	0	6 (60.0%)	2	0 (0.0%)	1	0.426
DM	1 (20%)	0	3 (25.0%)	0	1 (10.0%)	2	0 (0.0%)	1	0.853
Total cholesterol		5	3.6 (3.0-5.2)	5	4.7 (3.2-6.1)	5		2	0.371
HDL		5	0.9 (0.8-1.1)	6	0.9 (0.9-1.3)	5		2	0.583
Previous aorta surgery	0 (0.0%)	0	6 (50.0%)	0	0 (0.0%)		0 (0.0%)	1	0.034*
Rupture	0 (0.0%)	0	0 (0.0%)	0	3 (30.0%)	0	0 (0.0%)	0	0.193
Artery disease	1 (20%)	4	11 (91.7%)	0	4 (40.0%)	2	1 (50.0%)	1	0.002*
Smoking	3 (60%)	5	11 (91.7%)	1	6 (60.0%)	1	1 (50.0%)	1	0.387
Packyears	27 (27-27)	4	33 (20-50)	2	45 (0-53)	1	0 (0-0)	1	0.819
CRP		5		12	12.5 (12.0-65)	4		2	n/a
Diameter CT		5	57 (55-71)	0	60 (45-81)	1	46 (46-46)	1	0.397
Beta blocker	1 (20%)	3	8 (66.7%)	0	3 (30.0%)	2	0 (0.0%)	1	0.567
ARB / ACEi	2 (40%)	2	6 (50.0%)	0	3 (30.0%)	2	0 (0.0%)	1	0.789
Calciumblocker	0 (0%)	3	3 (25.0%)	0	0 (0.0%)	2	0 (0.0%)	1	0.439
Diuretics	0 (0%)	4	1 (8.3%)	0	2 (20.0%)	0	1 (50.0%)	1	0.009*

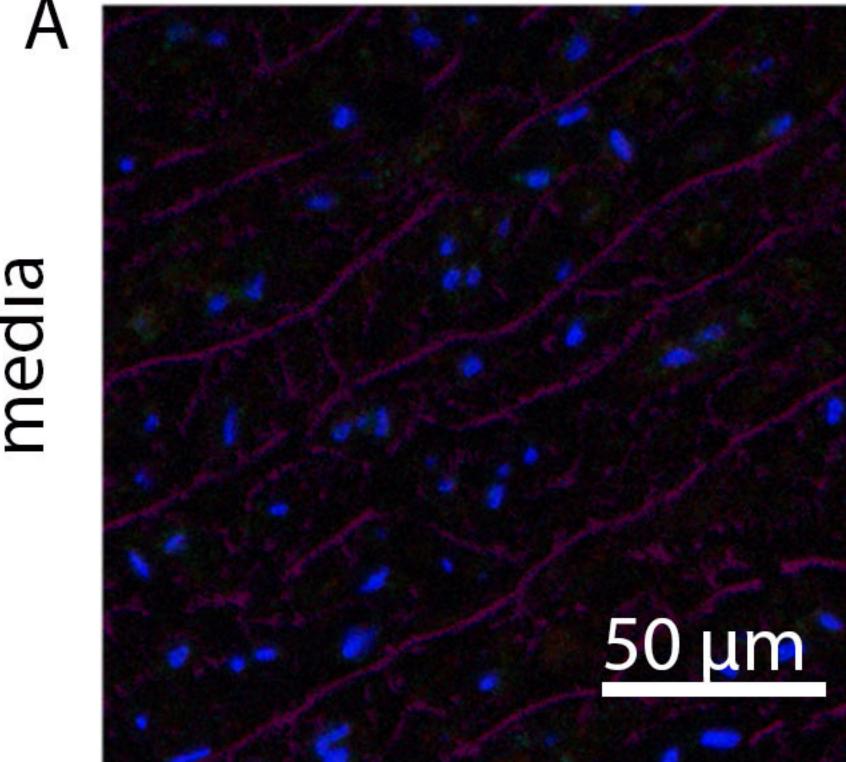
1 Table 1: Baseline characteristics. \* Represents  $P \le 0.05$ . Numbers display numbers of patients with percentage, or median with interquartile range (IQR).

	Adaptive immune system	า	Innate immune system	
Markers (clone)	DAPI		DAPI	
	CD3 (SP7)		CD68 (PG-M1)	
	CD8 (CD8/144B)		CD206 (CL038+)	
	CD20 (L26)		CD15 (MMA)	
	CD1c (2F4)		CD31 (JC70A)	
	FoxP3 (236A/E7)		MMP9 (polyclonal)	
	CD45RO (UCHL-1)		GM-CSF (polyclonal)	
	Autofluorescence		Autofluorescence	
Cell phenotype	T cell	CD3+	Macrophage	CD68+
	Helper T cell	CD3+ CD8-	M1-like macrophage	CD68+ CD206-
	Cytotoxic T cell	CD3+ CD8+	M2-like macrophage	CD68+ CD206+
	Regulatory T cell	CD3+ CD8- FoxP3+	Neutrophil	CD15+
	Memory T cell	CD3+ CD45RO+	Endothelium	CD31+
	B cell	CD20+	MMP9+ cell	MMP9+ CD15-
	Classic DC type 2	CD1c+ CD20-	MMP9+ neutrophil	MMP9+ CD15+
	DAPI	Nucleus	DAPI	Nucleus
	Autofluoresence	Elastin fibers	Autofluoresence	Elastin fibers

2 Table 2: Overview of the used markers and clones per panel, including definition of each cell type as used for our analysis.

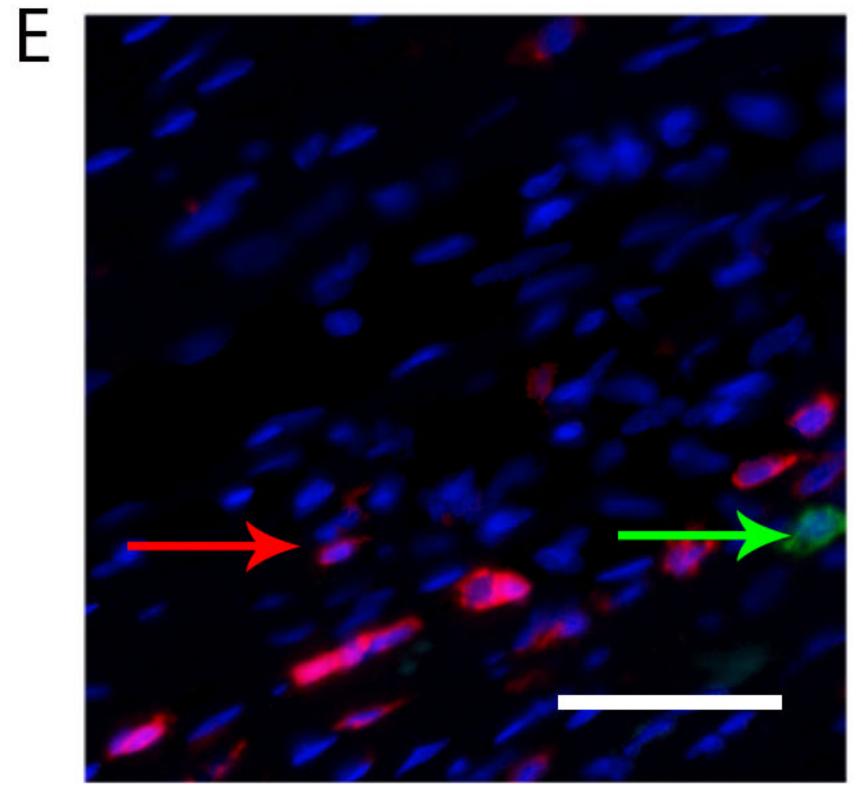


media

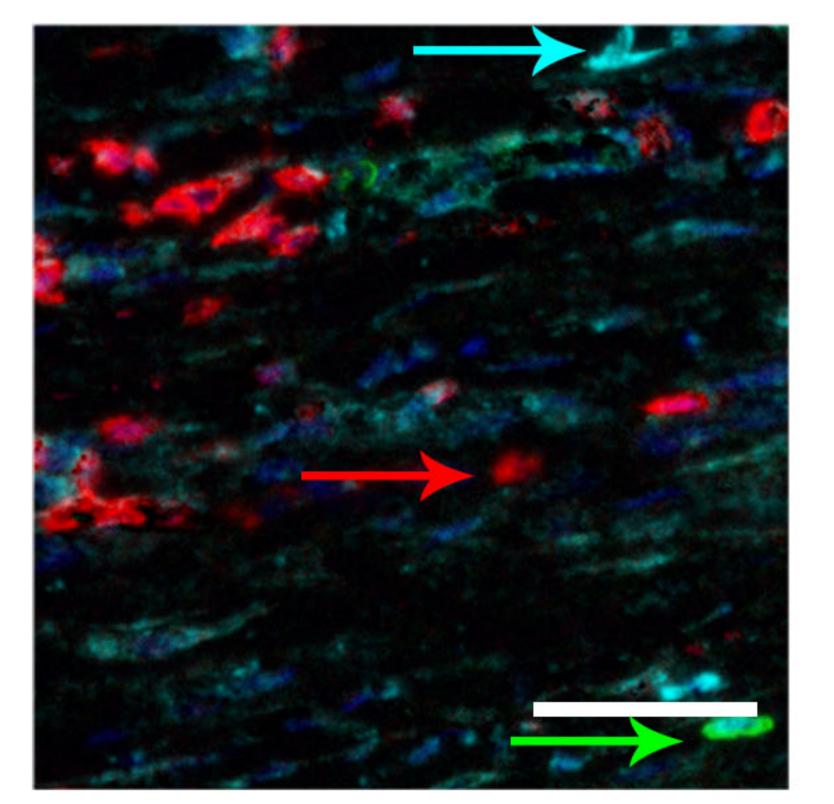


normal aorta

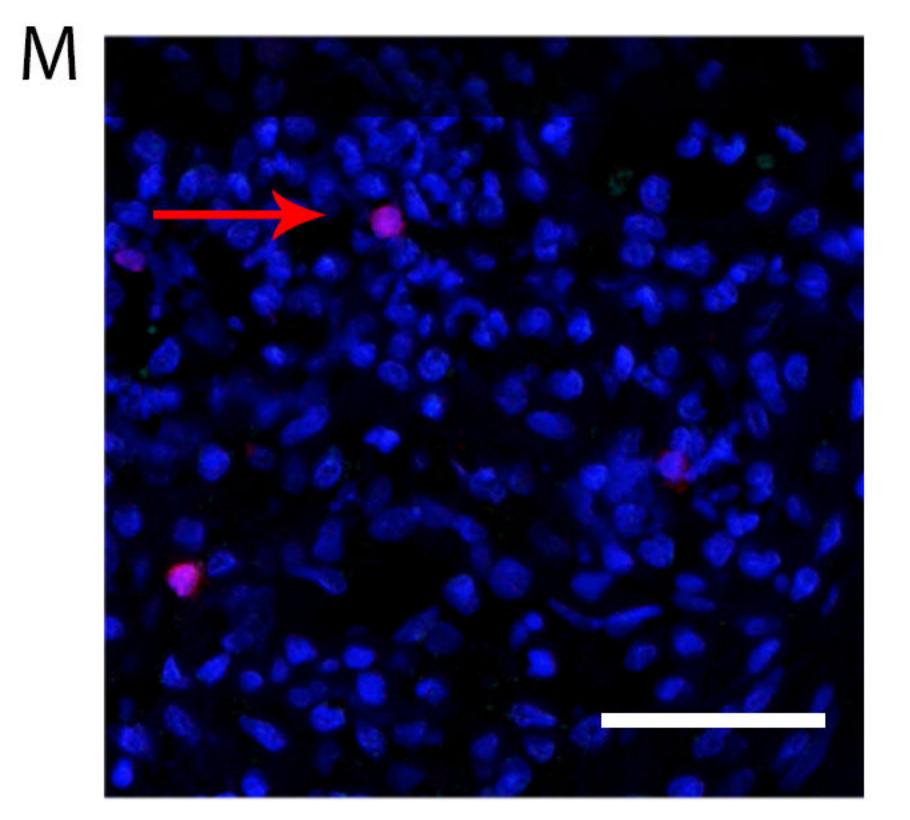
## atherosclerotic AAA



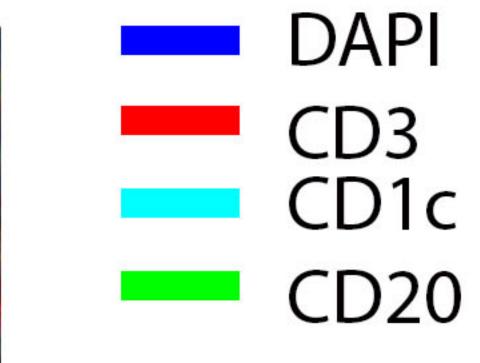
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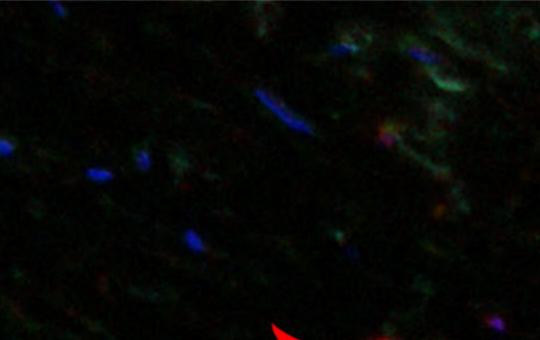


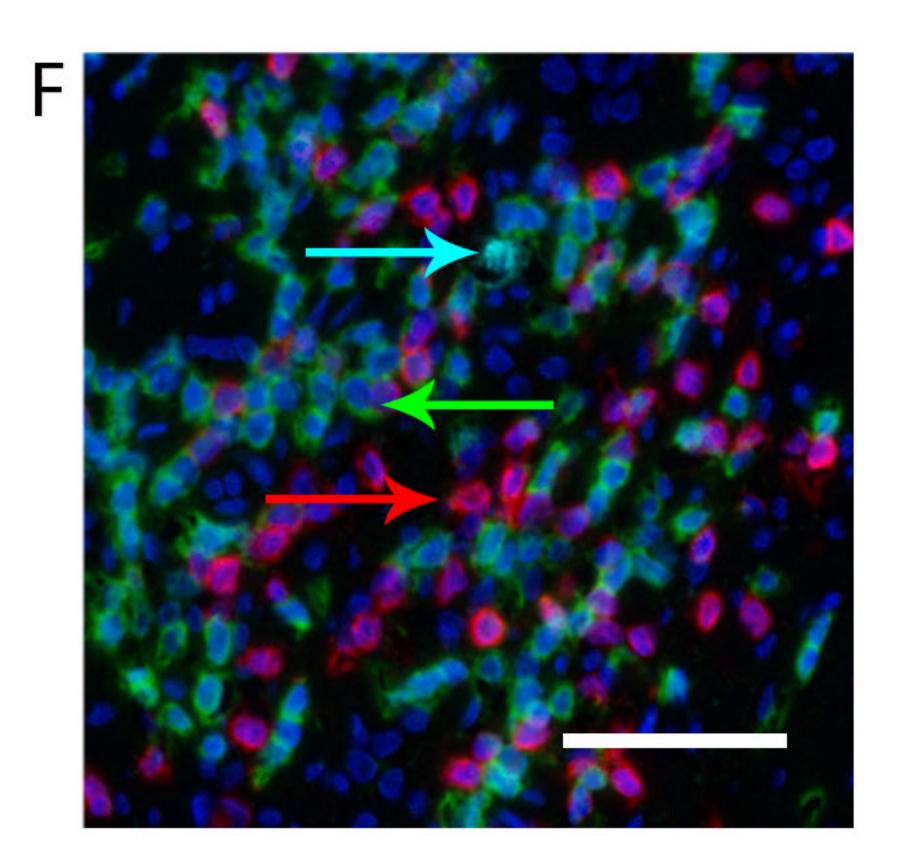
### acute infection of AAA

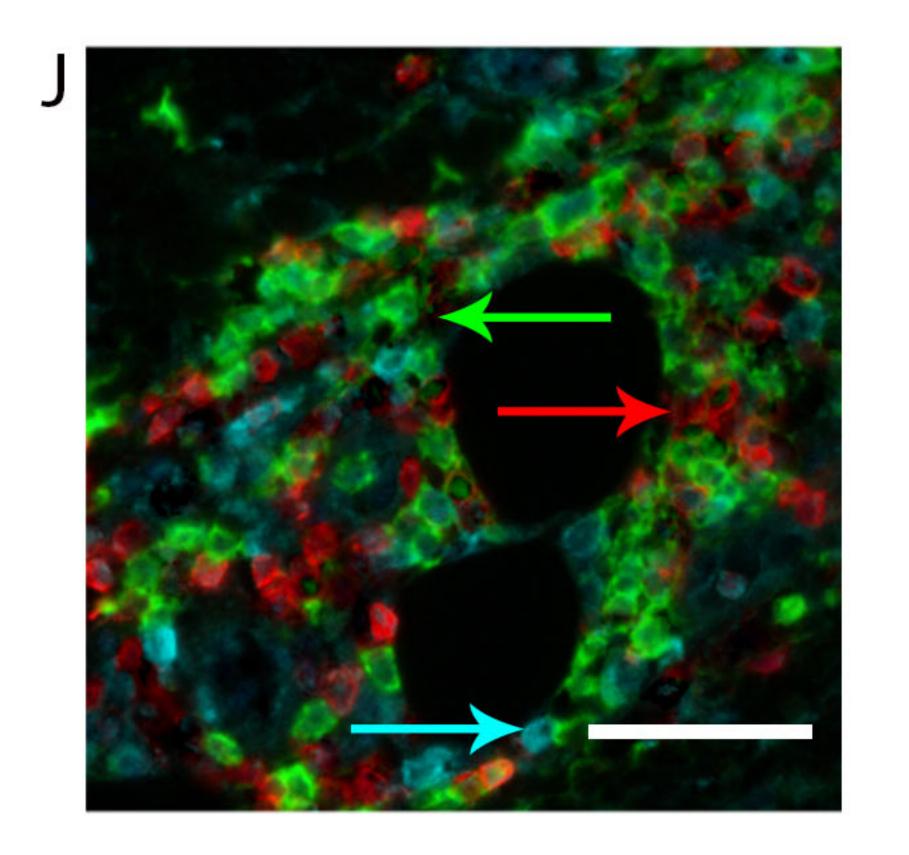


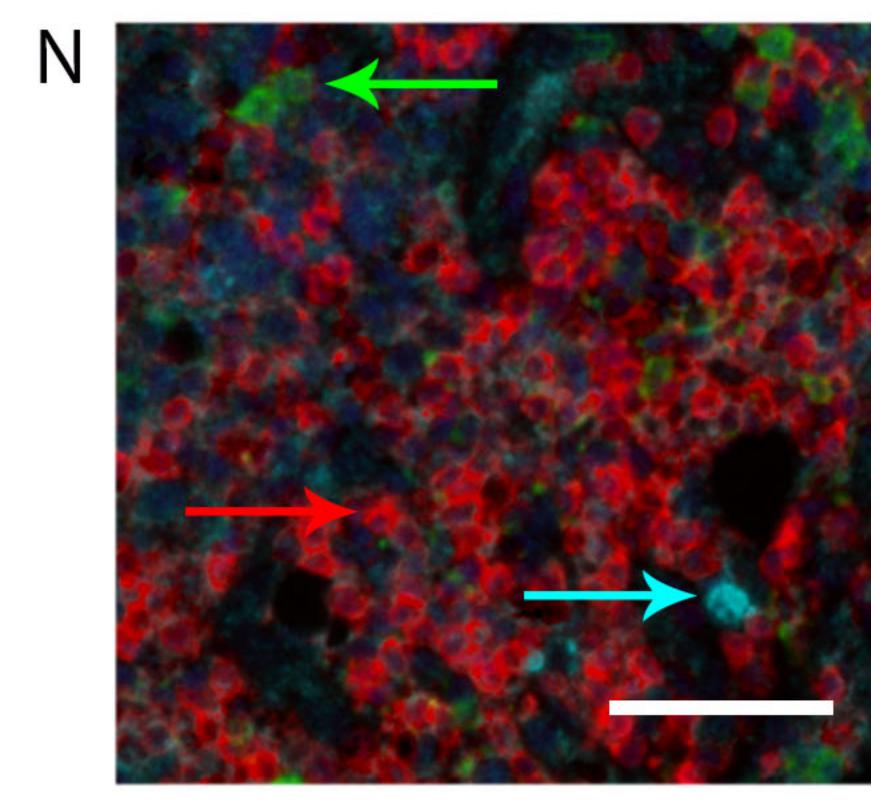
DAPI CD3 CD1c CD20





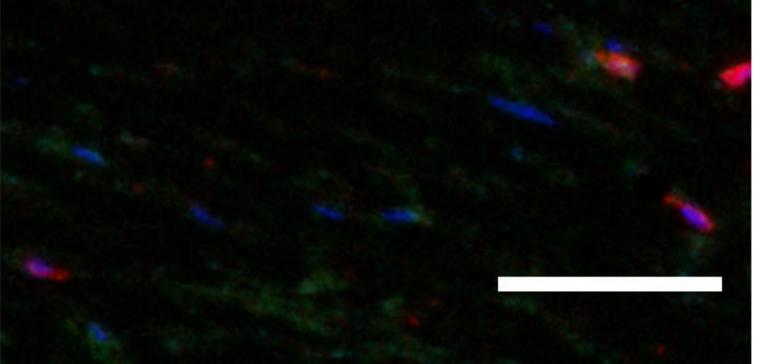




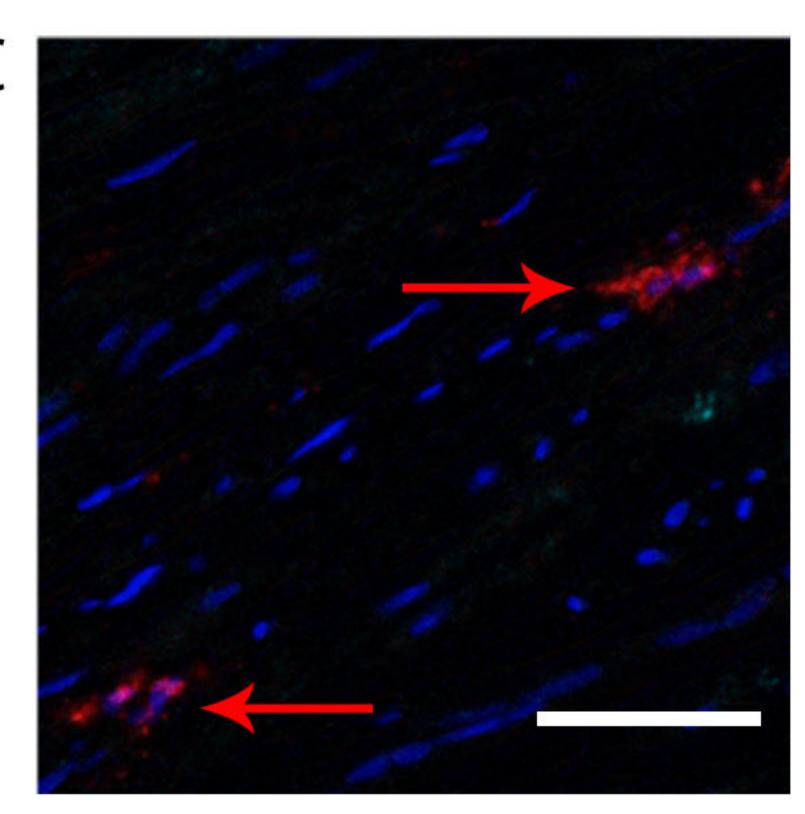


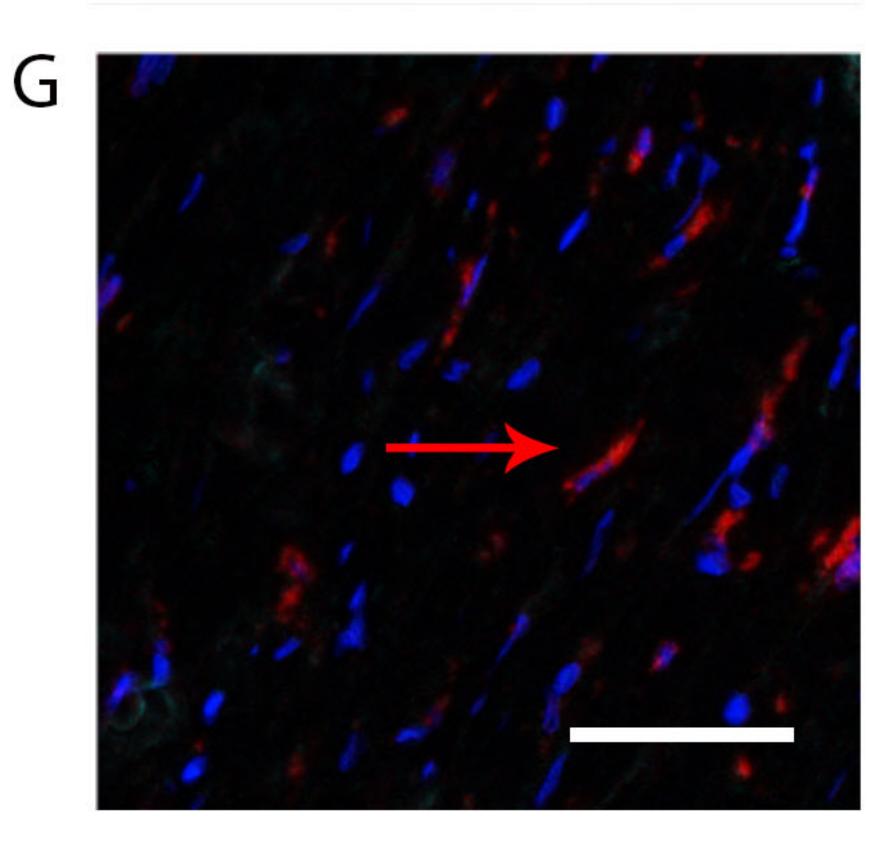
# adventitia

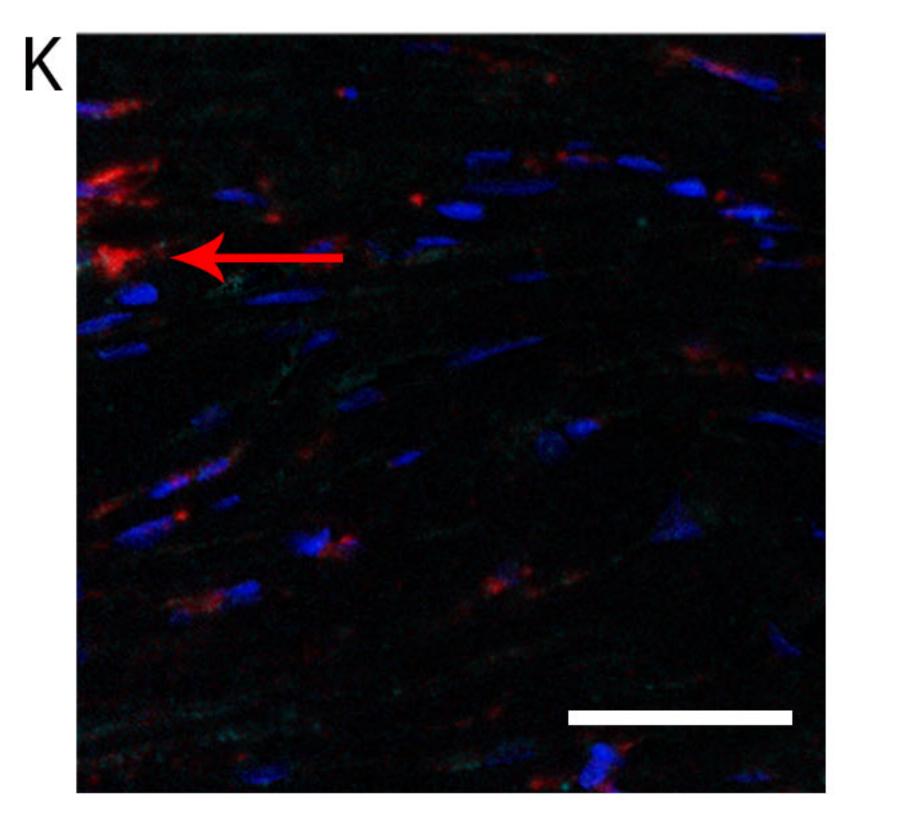
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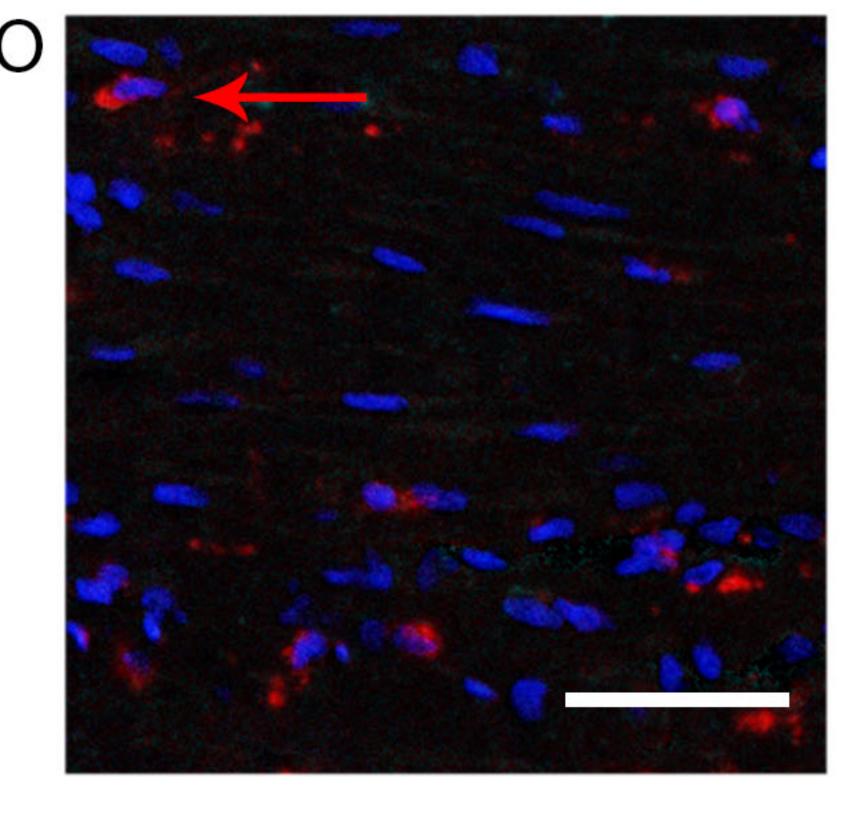






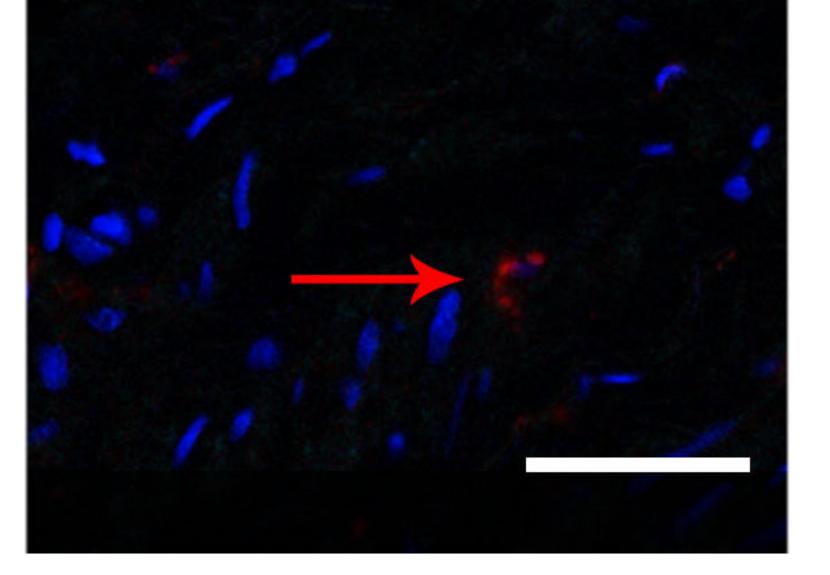


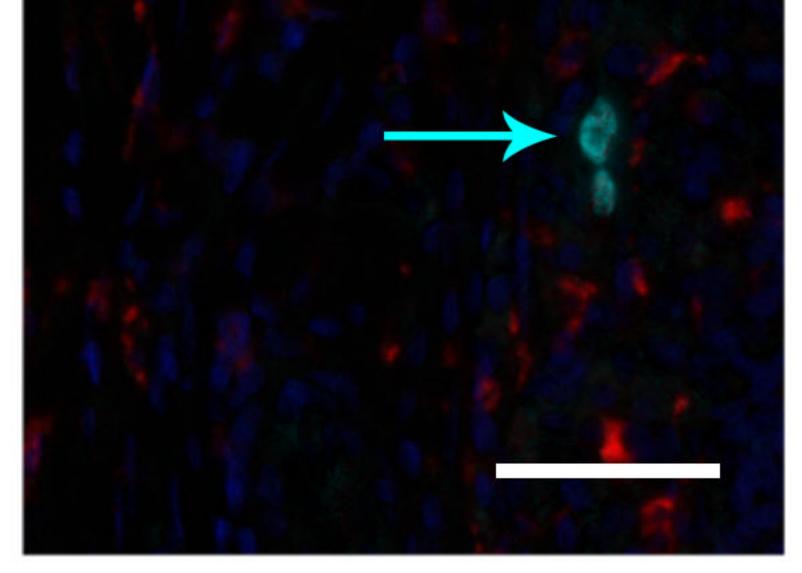




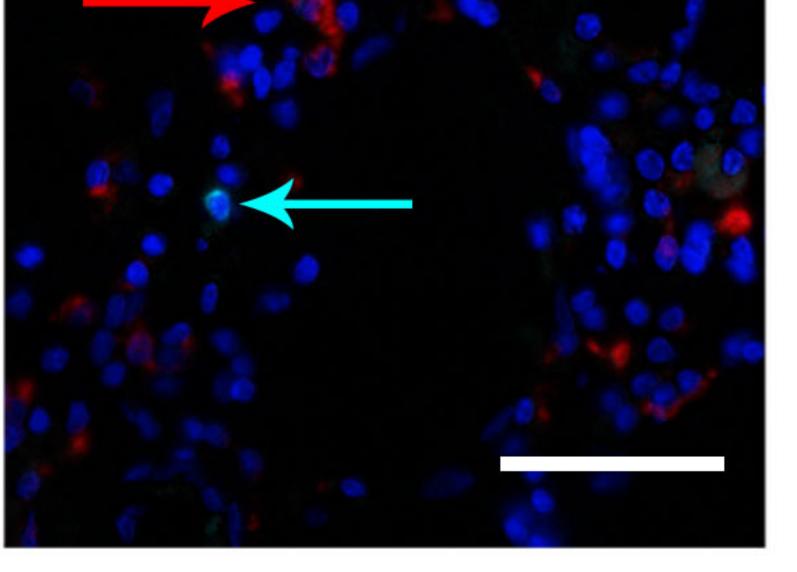
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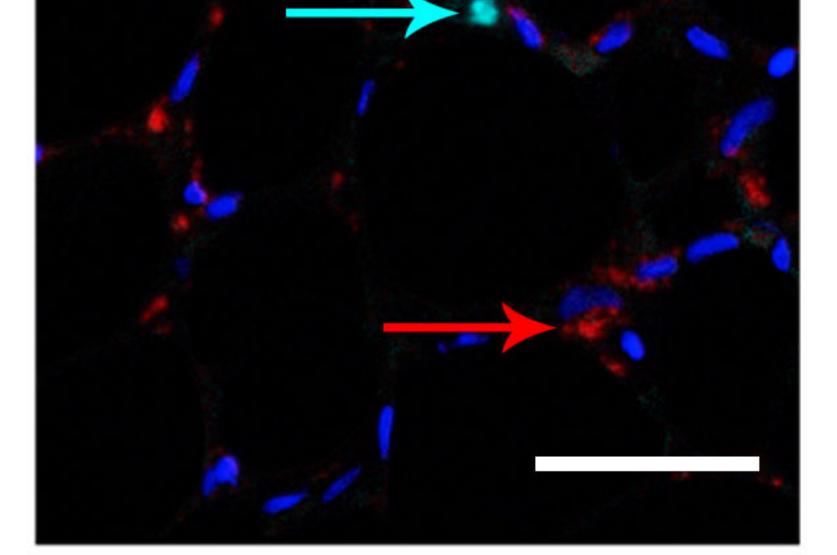




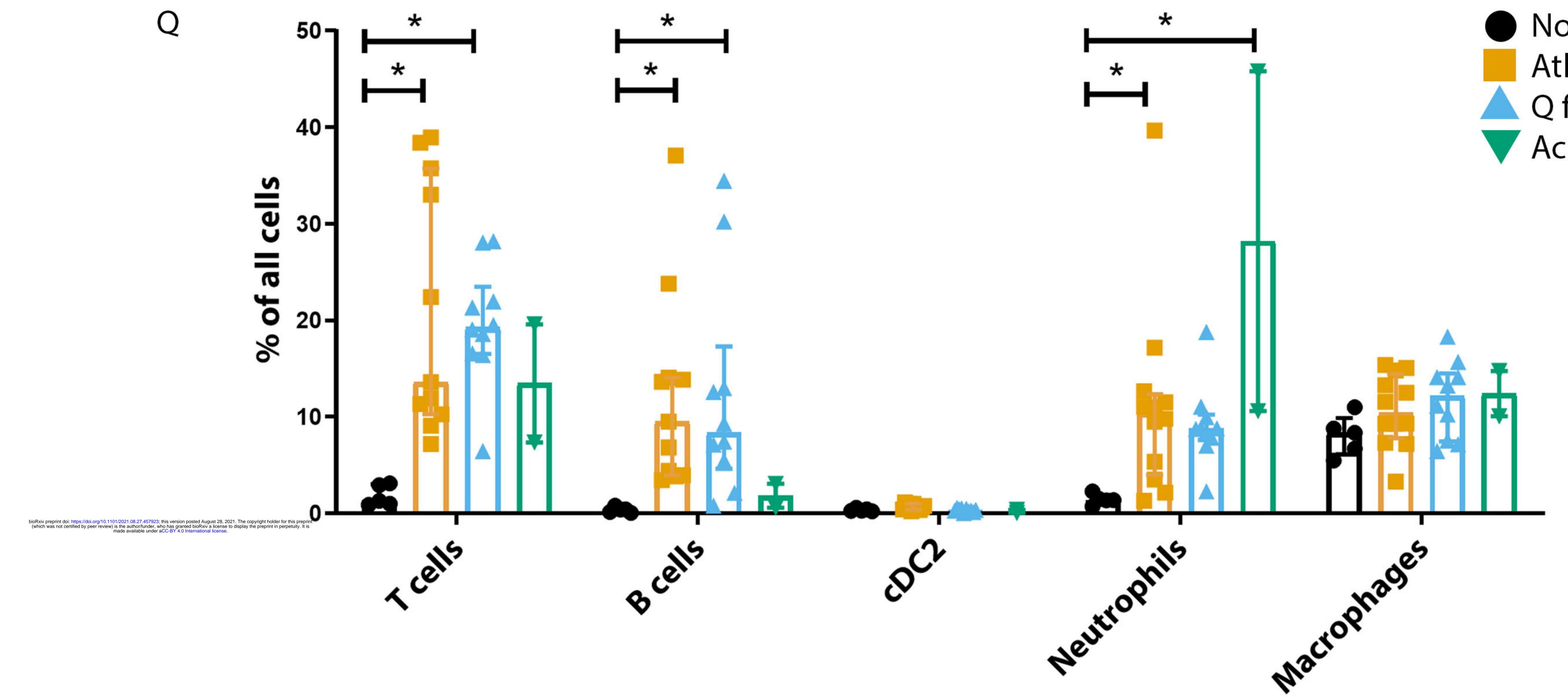


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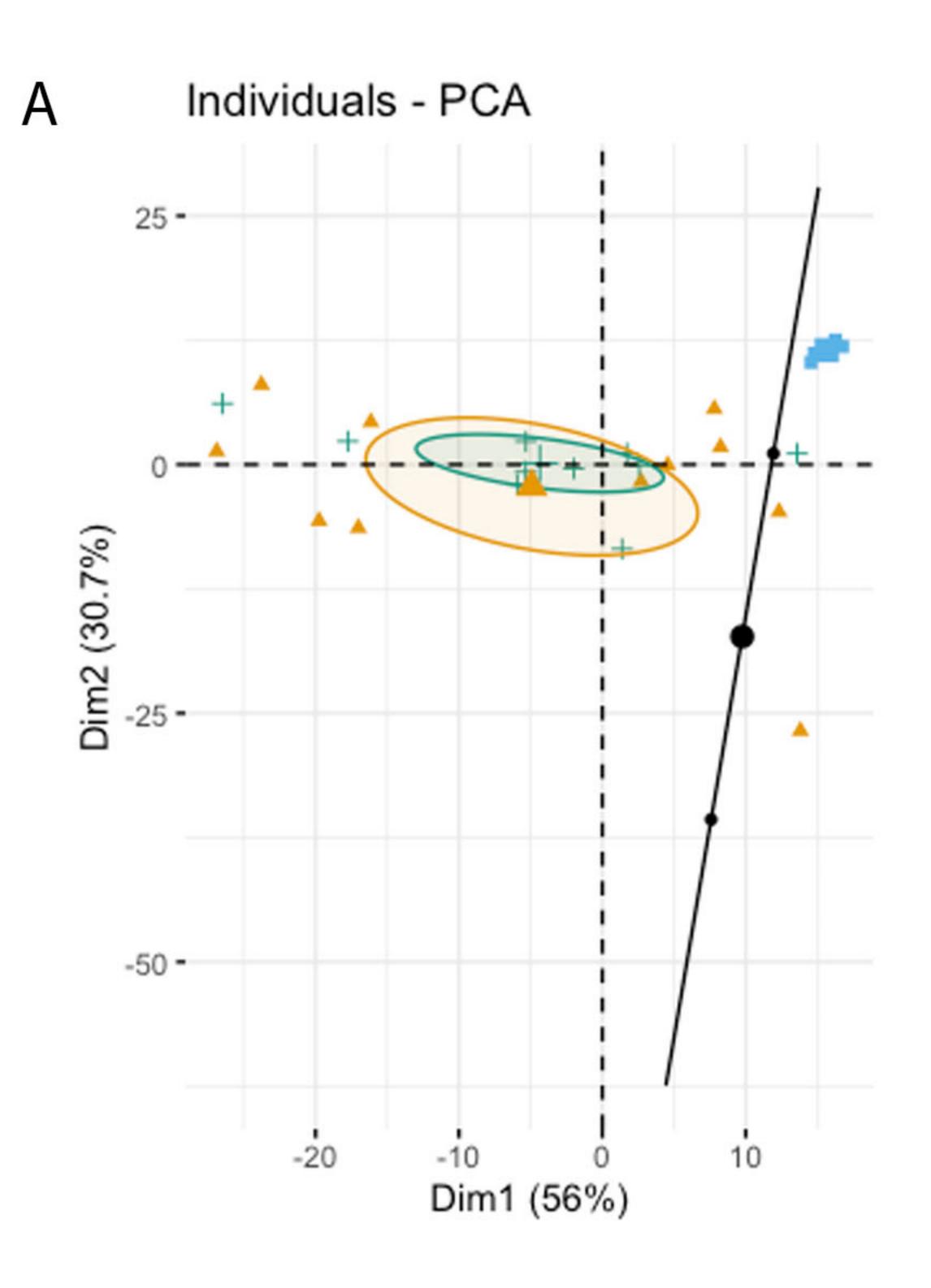


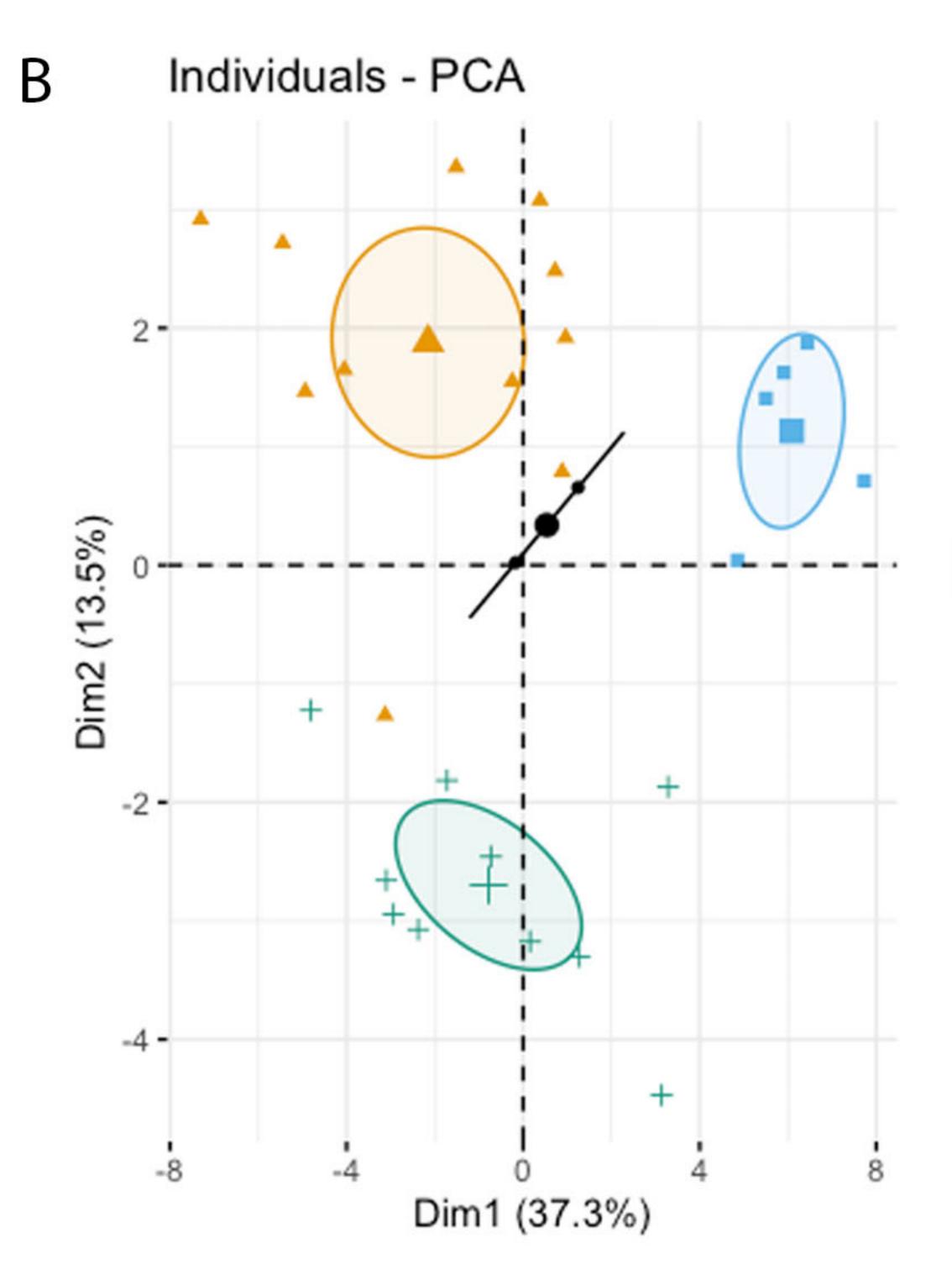


## Percentages of cells



Normal aorta Atherosclerotic AAA Q fever AAA Acute infection of AAA

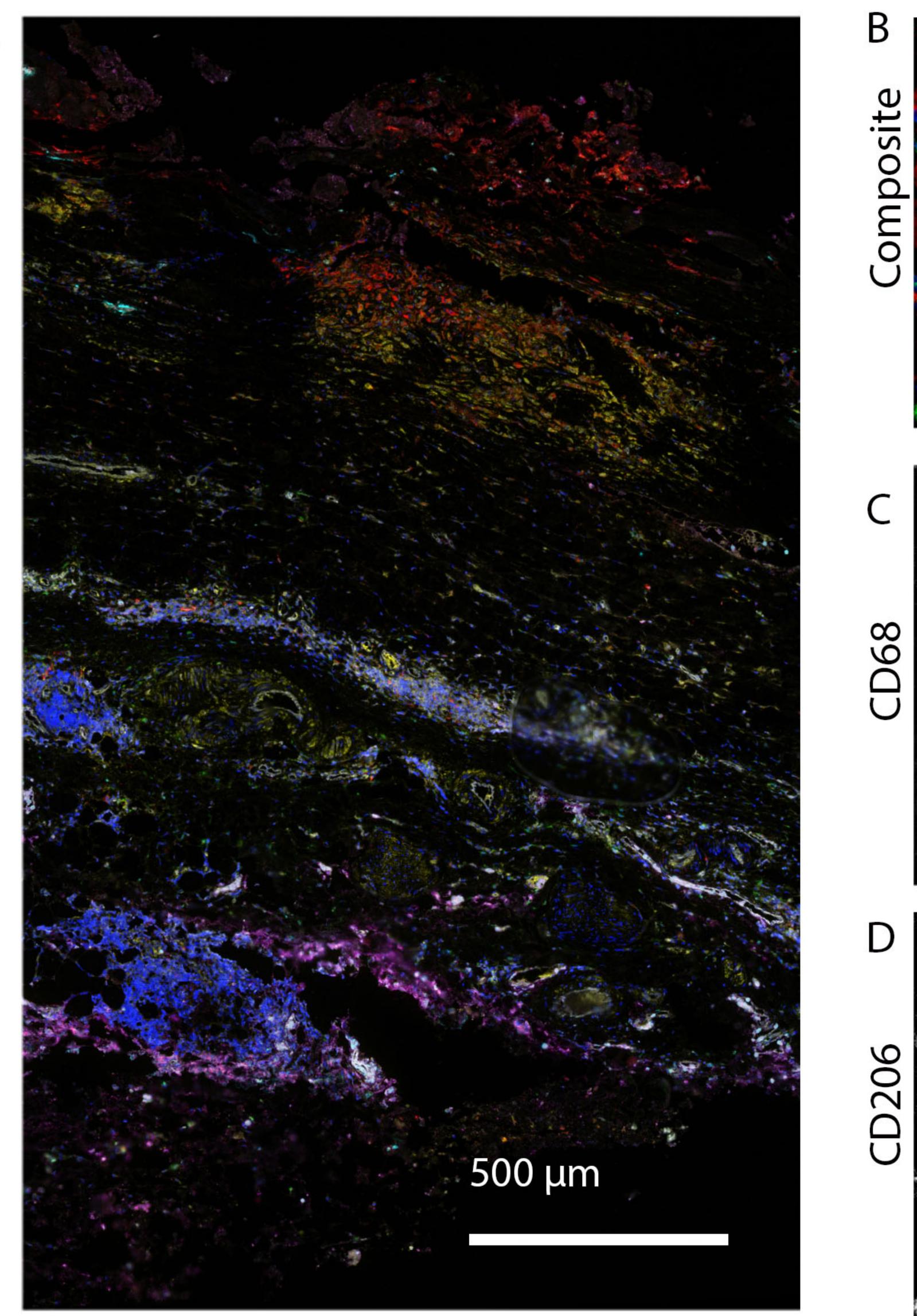


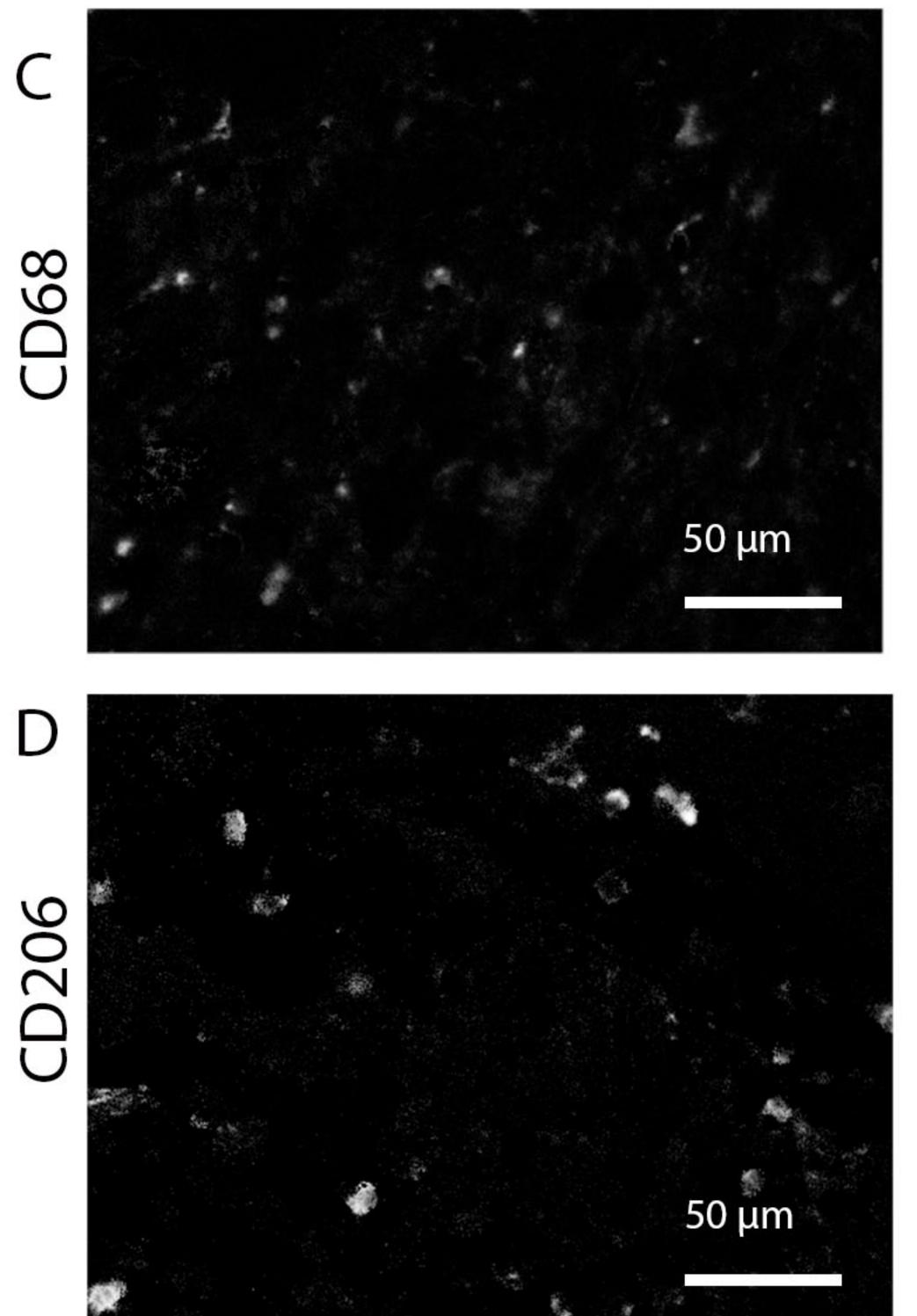


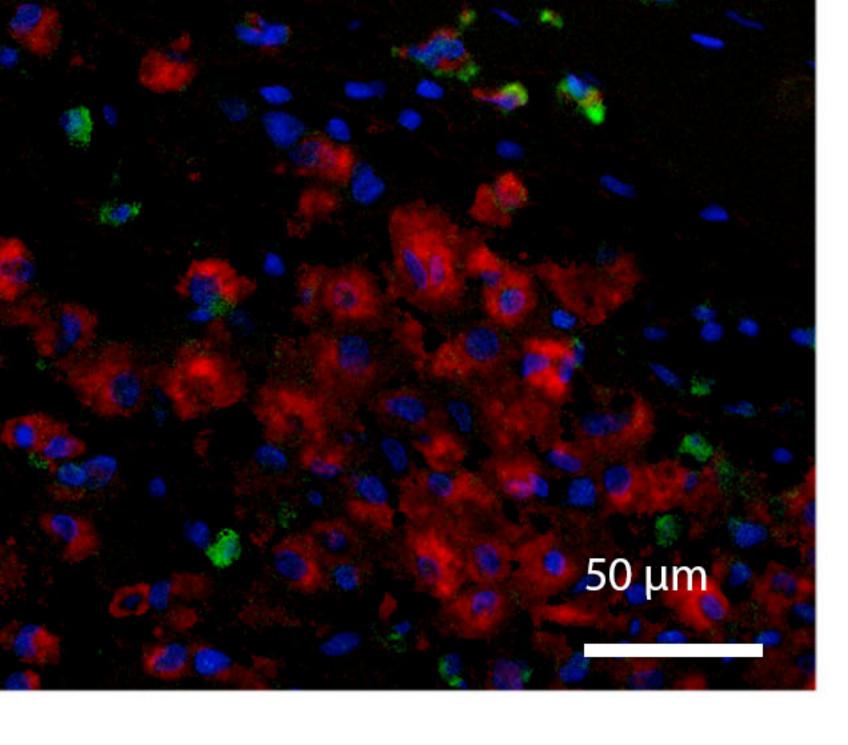
Groups



 Acute infection of AAA Atherosclerotic AAA Normal aorta + Q fever AAA

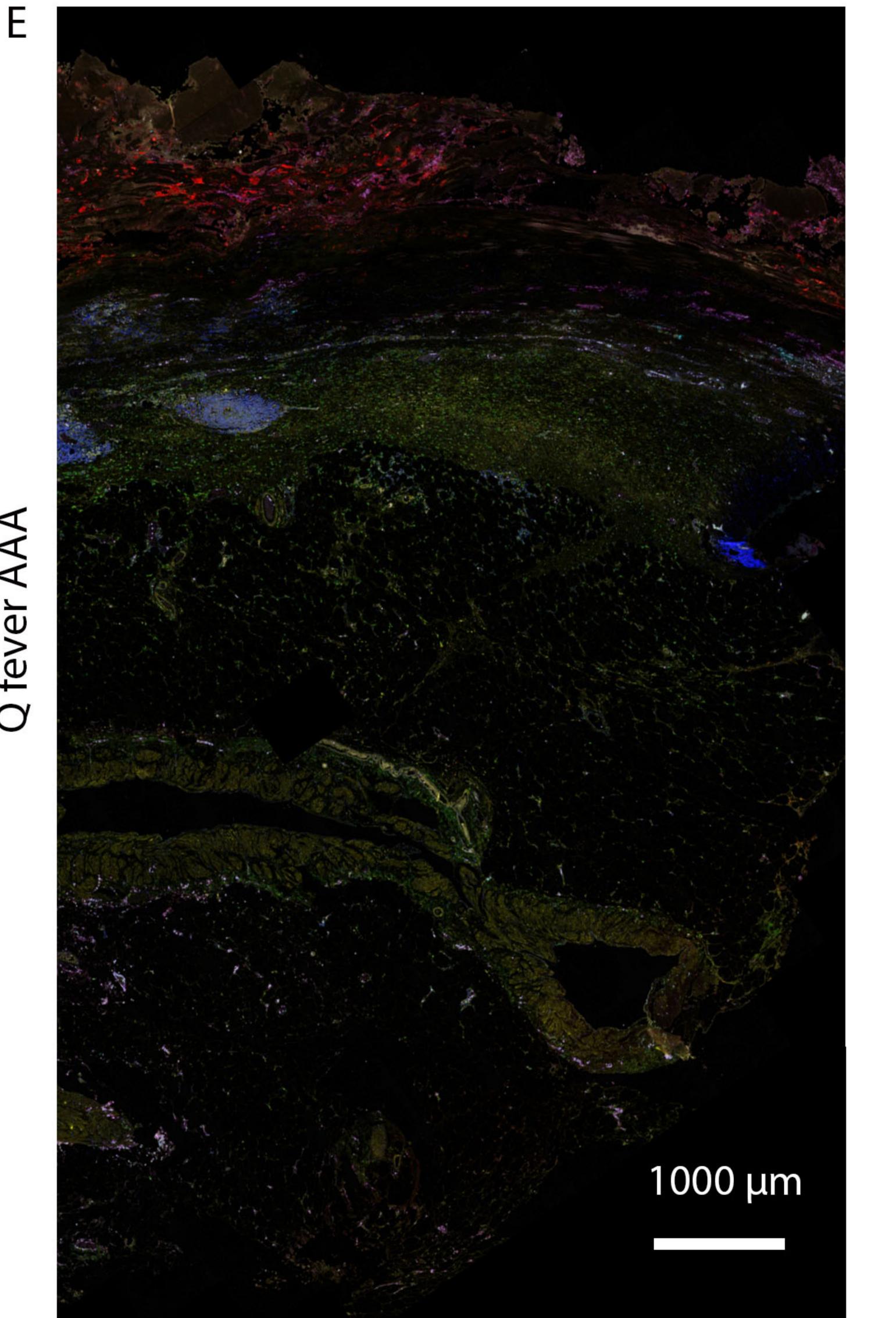


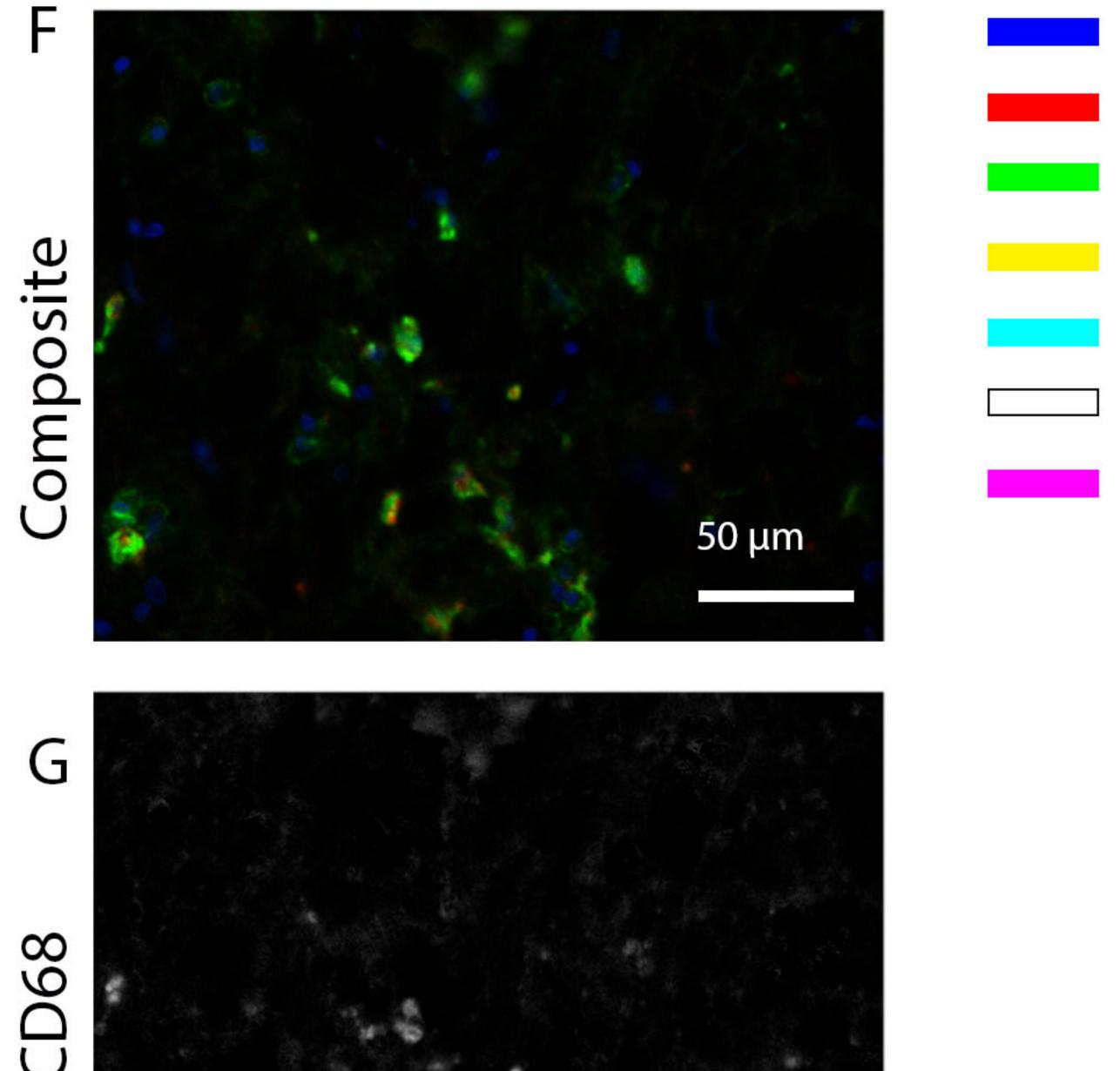






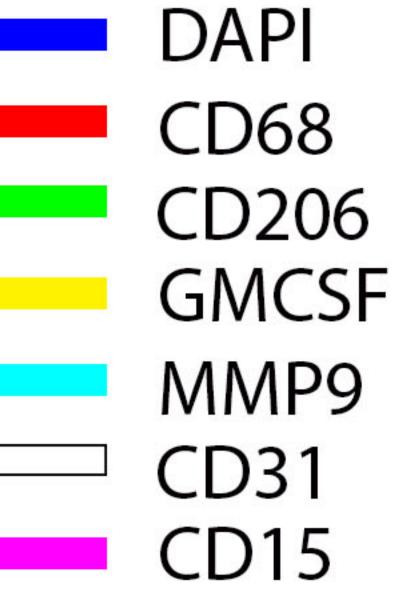
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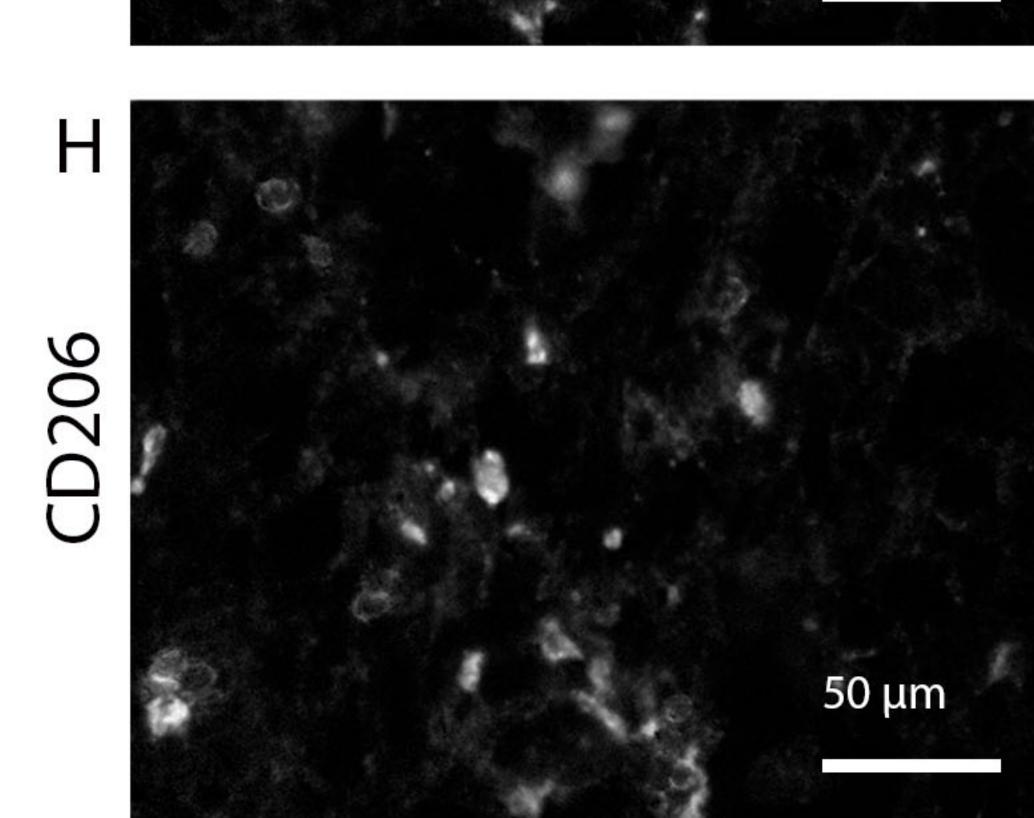




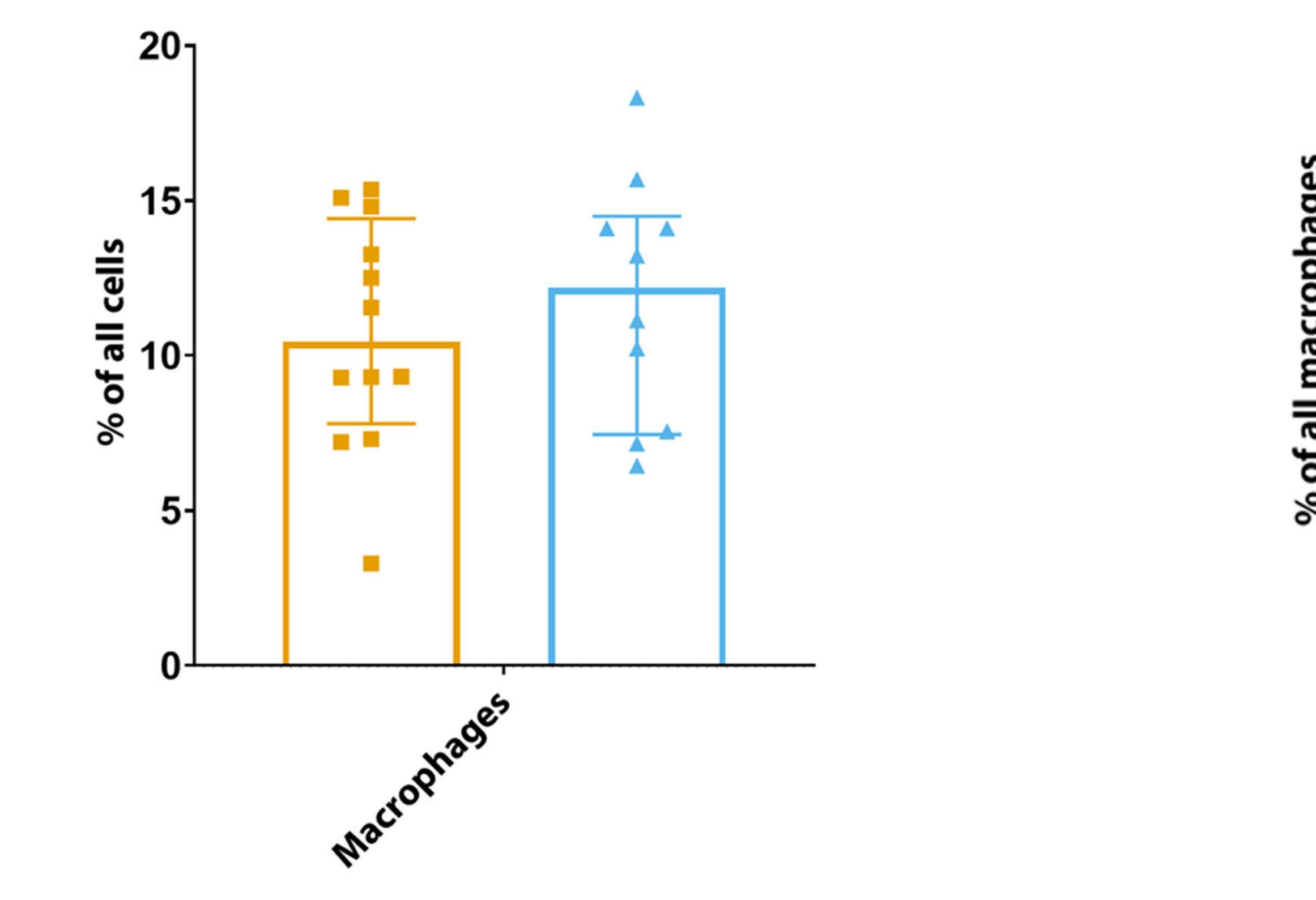
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## Percentages of macrophages

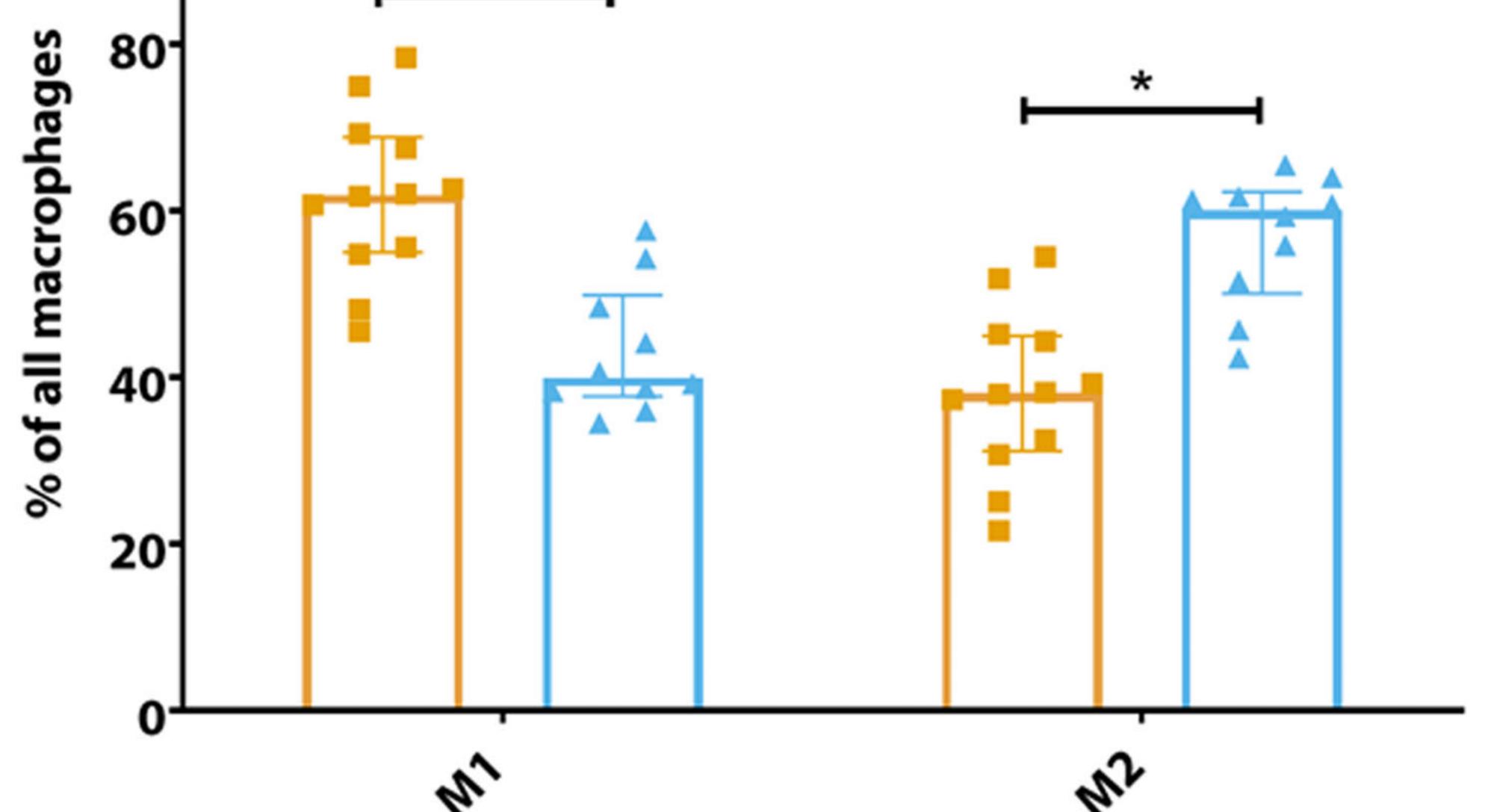


## Percentages of macrophage subsets

\*

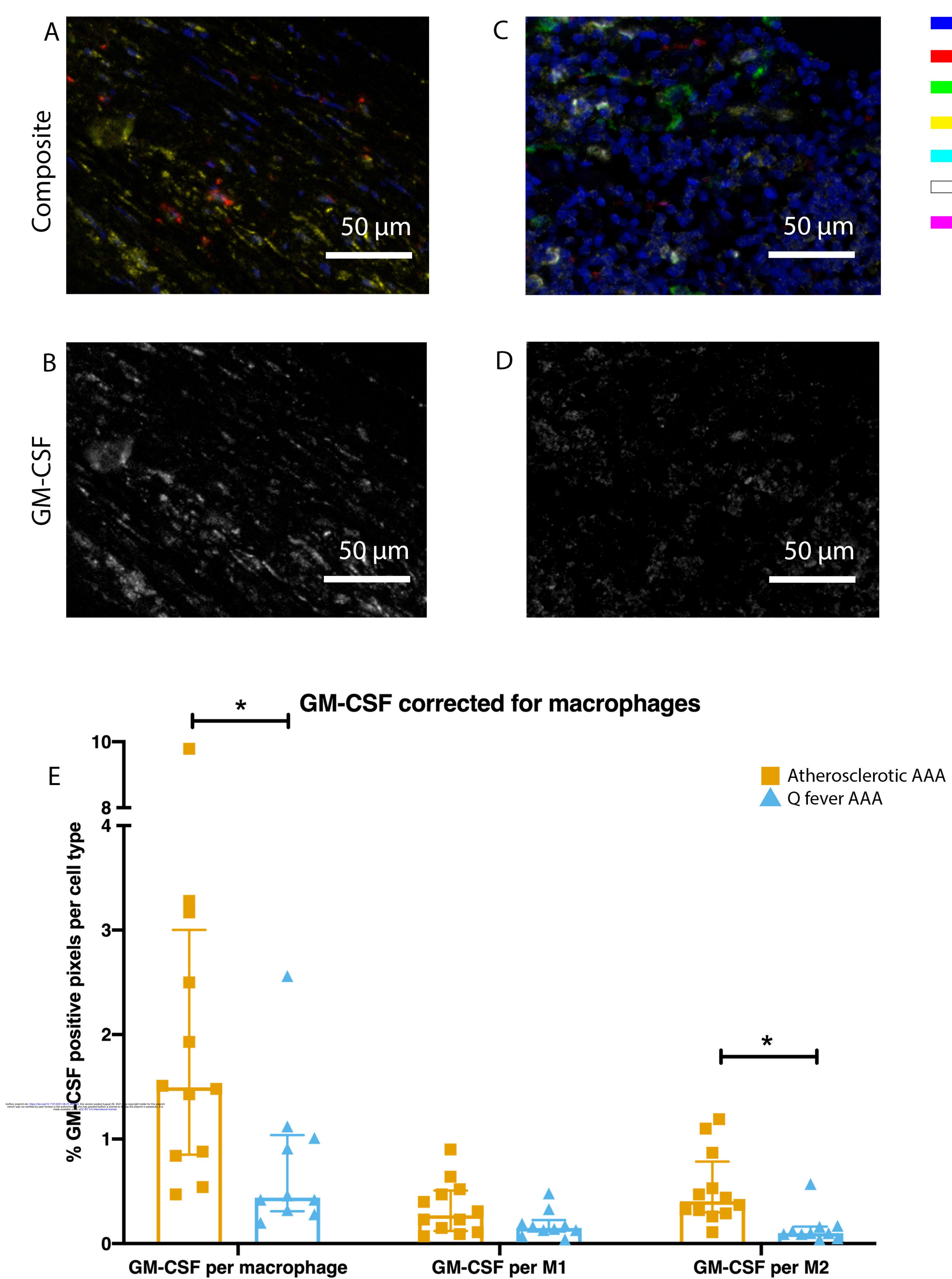


50 µm



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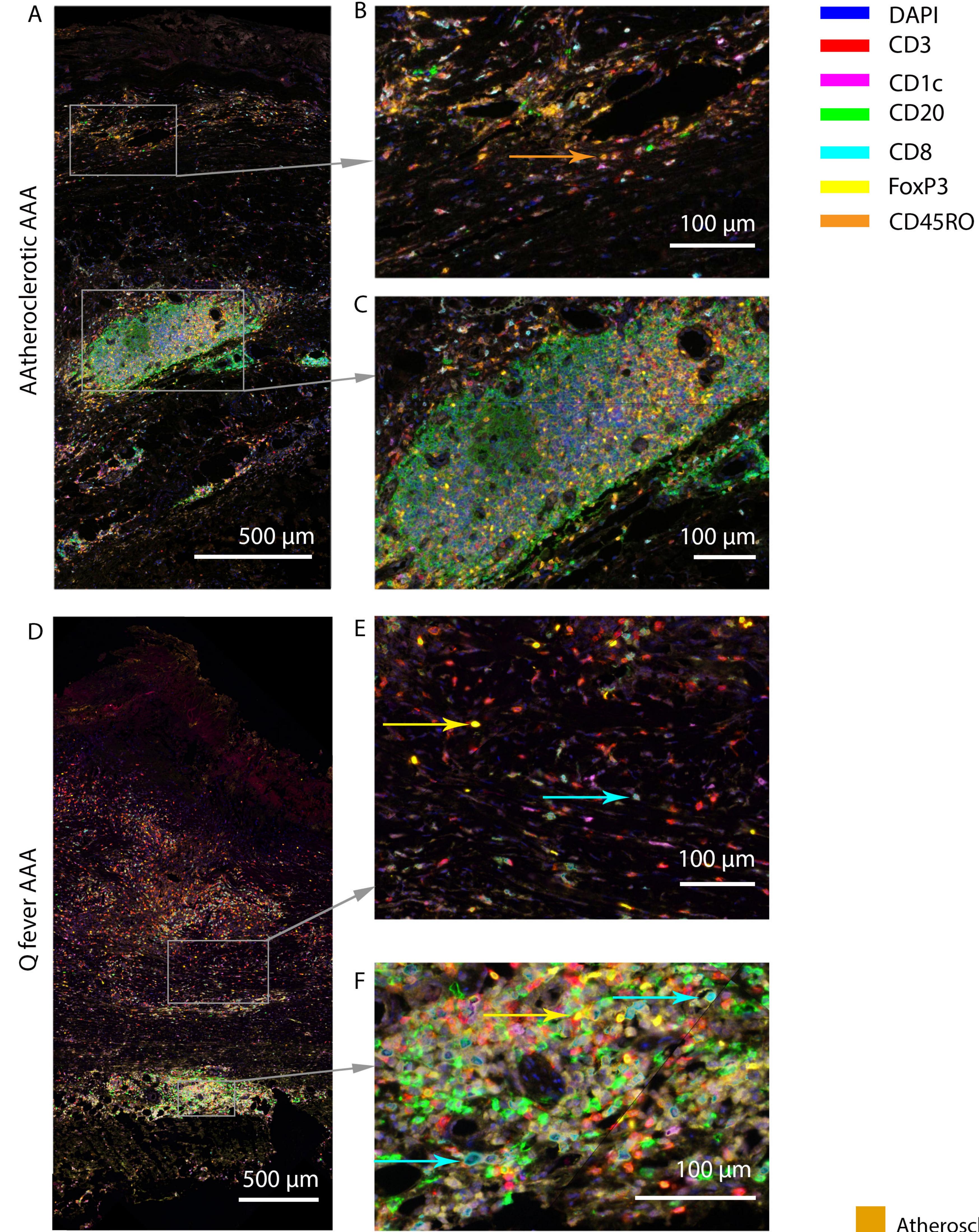
## Atherosclerotic AAA



GM-CSF per macrophage

## Q fever AAA

DAPI CD68 CD206 GMCSF MMP9 CD31 CD15





Atherosclerotic AAA



