Neutrophil infiltration to the brain is platelet-dependent, and is reversed by blockade of platelet GPIb

Neutrophil infiltration to the brain is platelet-dependent, and is reversed by blockade of platelet GPIbα

Short title: Platelets are needed for neutrophil infiltration in innate immunity

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Keywords: Inflammation, Brain, Neuroinflammation

Abbreviations:

CBA cytometric bead array

CNS central nervous system

IL-1 interleukin-1

LPS lipopolysaccharide

PBS phosphate buffered saline

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Summary

Neutrophils are key components of the innate immune response, providing host defence against infection and being recruited to non-microbial injury sites. Platelets act as a trigger for neutrophil extravasation to inflammatory sites but mechanisms and tissue-specific aspects of these interactions are currently unclear. Here, we use bacterial endotoxin in mice to trigger an innate inflammatory response in different tissues and measure neutrophil invasion with or without platelet reduction. We show that platelets are essential for neutrophil infiltration to the brain, peritoneum and skin, neutrophil numbers returning to basal levels in the peritoneum and skin and being decreased (~60%) in the brain when platelets are reduced. In contrast neutrophil infiltration in the lung is unaffected by platelet reduction, up-regulation of CXCL-1 (2.4-fold) and CCL5 (1.4-fold) acting as a compensatory mechanism in platelet-reduced mice during lung inflammation. In brain inflammation targeting platelet receptor GPIbα results in a significant decrease (44%) in platelet-mediated neutrophil invasion, whilst maintaining platelet numbers in the circulation. These results suggest that therapeutic blockade of platelet GPIbα could limit the harmful effects of excessive inflammation while minimising haemorrhagic complications of platelet reduction in the brain. The data also demonstrate the ability to target damaging brain inflammation in stroke and related disorders without compromising lung immunity and hence risk of pneumonia, a major complication post-stroke. In summary, our data reveal an important role for platelets in neutrophil infiltration to various tissues, including the brain, and so implicate platelets as a key, targetable component of cerebrovascular inflammatory disease or injury.

Introduction

The innate immune response provides rapid defence against infection, injury or disease. Neutrophils are the primary cellular response unit during the initial stages of these challenges and are essential for the destruction or removal of inciting stimuli (1). However, prolonged or excessive neutrophil mediated inflammation is injurious to adjacent healthy tissue in many situations, and is especially harmful during central nervous system (CNS) inflammation where capacity for repair is limited (2). An interaction with platelets is essential to trigger the tethering and rolling of neutrophils on inflamed venules, before their extravasation (3). Activated platelets attach to neutrophils via the release and surface expression of platelet P-selectin from α-granules which binds to PSGL-1 expressed on neutrophils (4). After CNS injury, a dense neutrophil invasion occurs (5-7), and selectively abrogating neutrophil infiltration is beneficial in animal models of stroke and experimental autoimmune encephalomyelitis (6,8). We have shown that mechanisms of neutrophil invasion following an innate immune challenge can be unique to their target tissue, allowing for tissue specific anti-inflammatory interventions (9). This may be especially important when targeting components of the immune system that are particularly susceptible to infections, such as after stroke and spinal cord injury (10,11). Here we assessed the contribution of platelets to neutrophil-mediated inflammation across a variety of tissue beds, to investigate tissue-specific mechanisms of innate immunity, as we have previously shown in the context of interleukin-1 (IL-1) (9). Furthermore, we investigated whether platelet-

dependent neutrophil infiltration could be blocked, without reducing platelet numbers and increasing the risk of haemorrhage after cerebral inflammation.

Materials and Methods

Animals

Experiments were performed on male 8-10 week-old wild-type (WT) C57BL/6 mice (Harlan Laboratories, Bicester, UK) under appropriate UK Home Office licences and adhered to the UK Animals (Scientific Procedures) Act 1986.

Inflammatory challenge

Peritoneal inflammation model: Mice were injected intraperitoneally with 1mg/kg lipopolysaccharide (LPS) from Escherichia coli O127:B8 (Sigma-Aldrich, Dorset, UK) or vehicle (phosphate buffered saline (PBS)) in a volume of 8 ml/kg. At 6 h peritoneal lavage was performed using 5 ml of lavage buffer (PBS containing 0.1% BSA and 1mM EDTA). Neutrophils in lavage fluid were quantified using Coulter Counter and haemocytometry measurements, combined with flow cytometry (see below).

Broncho-alveolar inflammation model: Mice were exposed to aerosolised LPS (2 mg/ml) or vehicle (saline) for 20 min via a nebuliser chamber. At 6 h broncho-alveolar lavage was performed, via direct cannulation of the trachea, with 1 ml of lavage buffer.

Air-pouch inflammation model: Dorsal air-pouches were created in conscious mice as described previously (12). At day 7, 1 ml of LPS (1 mg/ml) or vehicle

(PBS) was injected into the air-pouch. After 6 h air-pouch lavage was performed using 4 ml lavage buffer.

Cerebral inflammation model: Animals were anaesthetised with isoflurane (3%) in O_2 (200 ml/min) and N_2O (400 ml/min) and placed securely in a small animal stereotaxic frame (Stoetling, Illinois, USA). After craniotomy, mice were injected intracerebrally with 1 µl LPS (4 mg/ml), via a glass micro-needle (co-ordinates from bregma: anterior-posterior -0.0 mm, lateral -2.0 mm, ventral -2.5 mm. Rate = 0.5 µl/min). The micro-needle was left in situ for 2 min following the injection. Mice were transcardially perfused with saline at 6 h and brain tissue collected for cytometric bead array (CBA) analysis or perfuse-fixed (saline followed by paraformaldehyde 4%) at 24 h for tissue sectioning.

Platelet reduction: Mice were injected i.p with anti-CD41 antibody (1 mg/kg) or IgG isotype control (1 mg/kg) 24 h prior to inflammatory challenge. Tail vein blood samples taken at 0, 18 and 24h post-injection were analysed via flow cytometry to quantify circulating platelets. To determine the effect of the antibody on circulating leukocyte populations, cardiac blood sampled prior to sacrifice at 48h post-injection underwent flow cytometric analysis (see below) to quantify the populations of various leukocytes. Platelet numbers were reduced by (~70%; Figure 1A) with no significant effect on circulating leukocytes (data not shown). To block platelet-endothelium interactions without any reduction in platelets, an anti-GpIbα Fab fragment (p0p/B) or isotype control IgG were injected i.p (4 mg/kg) 4 h before intrastriatal injection of LPS. Anti-GpIbα treatment had no effect on numbers of circulating neutrophils (data not shown).

Flow cytometry

Lavage fluid (200 µl) or blood (50**µ**l blood + 50**µ**l buffer: 0.1% BSA, 1mM EDTA in PBS) samples were incubated for 20 min with 1:200 rat anti-mouse CD16/CD32 to block non-specific Fc binding. Cocktails of fluorophore-conjugated antibodies were added for 30 min, to detect Ly-6G, Ly-6C, CD45, B220, CD3, MHC-2, Gr-1, CD11b, CD115, CD41 and CD61. Red blood cells in samples were lysed by the addition of 450ml FACS Lysing Solution (BD Biosciences, Oxford, UK). Absolute numbers of cells were determined through the use of TruCOUNT™ tubes (BD Biosciences, Oxford, UK), or by the addition of 50 µl fluorescent counting beads (inVitrogen, Paisley, UK). Flow cytometry was performed on a CyAn™ ADP Flow Cytometer (Dako UK Ltd, Ely, UK) equipped with 405, 488 and 633 nm lasers using Summit 4.0 software. Cell populations were determined on Summit 4.0 software via positive labelling of relevant markers. For blood samples, a minimum of 1000 beads, 1,000 neutrophils or 5,000 leukocytes (whichever threshold occurred last) were acquired per sample. For lavage samples, a minimum of 20,000 cellular events were acquired per sample.

Immunostaining

Total cell numbers in brain tissue sections were determined via microscopy following immunohistochemistry staining. Anti-neutrophil (SJC4, rabbit anti-mouse) primary antibody (1:50,000; kindly provided by Professor Daniel Anthony, University of Oxford, UK) was used to stain for neutrophils. Neutrophils were quantified in three regions (cortex, injection site and ventral

striatum) using a 10x10 mm graticule at 20x magnification. Cerebrovascular activation was determined by the expression of vascular cellular adhesion molecule-1 (VCAM-1, goat anti-mouse primary antibody, 1:250; R&D systems, UK).

Cytometric bead array

Cytokine concentrations in plasma and lavage samples were determined using mouse-specific CBA flex sets (BD Pharmingen, UK). CBA was used to quantify IL-1α, IL-1β, IL-6, TNFα, CXCL1, and CCL5 following the manufacturer's recommended protocol. Acquisition was undertaken using a BD FACSArray™ Bioanalyzer System (BD Biosciences, UK), and results determined using FCAP Array™ software (Soft Flow, Minnesota, USA).

Statistics

Data are expressed as mean (± sem). Differences between groups were analysed using one-way ANOVA with Bonferroni's correction for multiple comparisons post-hoc. Differences were considered statistically significant at $p<0.05$.

Results

Platelets are essential for neutrophil invasion to the peritoneum, skin and brain, but not lung after LPS-induced inflammation

Until recently, the precise mechanism of platelet-neutrophil interaction *in vivo* during innate immune responses was unclear. However, Sreeramkumar and co-

workers recently described platelet-neutrophil dynamics in inflamed cremaster blood vessels, showing platelets are key to initiating the process of neutrophil tethering, rolling and crawling upon vessels (3). To determine if platelets are required to drive innate immune responses in other tissues we stimulated inflammation with the bacterial endotoxin, LPS, in mice with a reduction in platelets. We used the accumulation of neutrophils as a measure of the intensity of the inflammatory reaction and assessed this at time-points coinciding with peaks of neutrophil influx established previously for the respective tissues (9). LPS stimulated significant increases in neutrophil accumulation in lavage fluid from the peritoneum (4-fold), air-pouch (~3-fold), lung (~13-fold) and in the brain (~23-fold) (Figure 1). In contrast, after platelet reduction, neutrophil recruitment was completely blocked in peritoneum (96%; Figure 1B) and air-pouch (100%; Figure 1C), and significantly reduced (66%) in the brain (Figure 1D), showing platelet dependent neutrophil infiltration for the first time in these tissues.

Redundancy of innate immune response mechanisms is an evolutionary advantage to tissues exposed to a wide variety of pathogens, such as the lung. Here, in contrast to peritoneum, air-pouch and brain, which are exposed to lower pathogenic load, we saw no effect of platelet reduction on neutrophil invasion after LPS-induced inflammation in the lung (Figure 1E), showing platelet-independent mechanisms of neutrophil invasion.

LPS injection in the peritoneum resulted in a significant increase (~2.5 fold) in the number of circulating neutrophils at 6h after injection, with anti-CD41 treatment having no effect on this increase (data not shown). In contrast LPS

when administered in the air-pouch, lung and brain did not affect circulating neutrophil numbers at 6h (data not shown).

Upregulation of CXCL1 and CCL5 in the inflamed lung counterbalance the effects of platelet reduction

To further investigate tissue specific-mechanisms at each site of inflammation, the cytokines IL-1α, IL-1β, IL-6, TNF and chemokines CXCL1 and CCL5, which are important for neutrophil recruitment (14), were measured with or without platelet reduction. LPS induced a similar profile of inflammation across all tissue sites (Figure 2). The inflammatory mediators measured were unaffected by platelet reduction in the peritoneum, air-pouch and brain (Figure 2). In contrast, with platelet reduction during lung inflammation, which failed to attenuate neutrophil recruitment, we found significant increases in the neutrophil chemoattractant CXCL1 (2.4-fold), and CCL5 (1.4-fold) in lung lavage fluid in animals with reduced platelets compared to IgG controls, and a non-significant increase in the pro-inflammatory cytokine TNF (Figure 2).

Targeting of platelet GPIbα reduces neutrophil infiltration to the brain

The innate immune system is essential for host defence but excessive or prolonged neutrophil-mediated inflammation is also associated with injury and disease, especially in the brain (2). The above data provide evidence for platelet-dependent neutrophil infiltration in three different tissue beds. However, depleting platelets as an approach to reduce inflammation has important limitations due to increasing vulnerability to haemorrhage. Therefore, to assess

whether platelet-dependent neutrophil infiltration could be blocked, in a manner with less potential systemic effects and therefore more relevant therapeutically and without reducing platelet numbers, we administered anti-GpIbα antibody in the context of LPS-induced brain inflammation. Anti-GpIbα antibody has been shown previously to protect mice from ischemic brain injury in an experimental stroke model without an increase in bleeding complications (15). Here, anti-GpIbα antibody had no effect on numbers of circulating platelets compared to IgG-injected controls (Figure 3A) yet significantly reduced (44%) the number of neutrophils in brain tissue after LPS (Figure 3B-C). We saw no effect of the anti-GpIbα antibody on endothelial activation, as assessed by VCAM-1 staining (Figure 3C).

Discussion

Neutrophil invasion to the brain is significantly reduced in mice lacking IL-1 (9), as it is here in mice with reduced numbers of platelets. Furthermore, we have shown previously that platelet-derived IL-1 drives endothelial activation *in vitro,* suggesting a convergence of brain and platelet-derived IL-1 effects on the endothelium.

These data replicate findings from Sreeramkumar et al., where only LPS plus an anti MHC-I antibody produced a strong enough lung inflammation to be attenuated by platelet intervention, as LPS-induced inflammation alone was unaffected by blocking of platelet activity (3). Together, these findings provide further evidence for tissue specific-mechanisms of innate immunity and highlight the flexibility of the lungs in dealing with pathogen-driven inflammation.

These data suggest the implementation of compensatory mechanisms specific to the lung in the absence of platelets, which would be advantageous to a site of such pathogenic exposure and explain the maintenance of neutrophil recruitment, despite platelet reduction.

Targeting neutrophil invasion during CNS injury may have therapeutic benefit as we, and others, have shown neutrophil mediated neurotoxic effects after stroke (6,16). Indeed, neutrophils have neurotoxic effects on neurones *in vitro* (17), an effect that appears dependent on phenotypic changes that occur during neutrophil cerebrovascular infiltration (18). Targeting various aspects of platelet activation reduces stroke injury (3,15,19). This, together with platelet-dependent neutrophil infiltration mechanisms (3) and the relevance of these in different tissues shown here, suggests that platelet targeted therapies may be beneficial after CNS injury.

A potential limitation of any immune-modulatory approach to treating acute CNS inflammatory conditions is the potential for increased risk of systemic infectious complications, notably in conditions such as stroke and head trauma. Pneumonia is the most common cause of infection in these patients and since the innate immune response in the lung is platelet-independent, in contrast to the brain, this may offer a relatively targeted approach to inhibiting damaging CNS inflammation without overly compromising respiratory anti-microbial innate immunity. Platelets adhere to hypoxic endothelial cells by binding of their GpIbα receptor to von Willebrand factor on the endothelial surface (20). Targeting this interaction may limit the time platelets spend at the endothelium and reducing the number of physical interactions with neutrophils, whilst concomitantly

reducing the ability of platelets to activate the endothelium, which is partly responsible for driving cerebrovascular inflammation (13). Therefore, targeting of the platelet GpIbα receptor is a potential therapeutic strategy for reducing neutrophil-mediated CNS injury.

In conclusion we show here that platelets are essential for neutrophil extravasation to inflammatory sites, but that this is dependent on specific tissue location. We show that platelets are essential for neutrophil infiltration to the peritoneum, skin and brain, but not the lung, where compensatory mechanisms allow for greater flexibility when dealing with pathogen. We also specifically show that platelet-mediated neutrophil invasion to the brain is dependent upon the receptor GPIbα which can be targeted to limit excessive inflammation while retaining platelet numbers and reducing the risk of haemorrhage in the brain.

Acknowledgements

JG, AG, BM, SA conceived of the study and designed experiments. JG, AG, AD, GC, BM performed experiments and analysed data. BN provided essential tools, reagents and expertise. BM, SG co-ordinated the study. AG, BM, SA wrote and edited the manuscript. All authors read and approved the final manuscript. We thank Professor Daniel Anthony (University of Oxford, UK) for kindly providing the SJC4 antibody. This work was funded by the British Heart Foundation and Medical Research Council.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

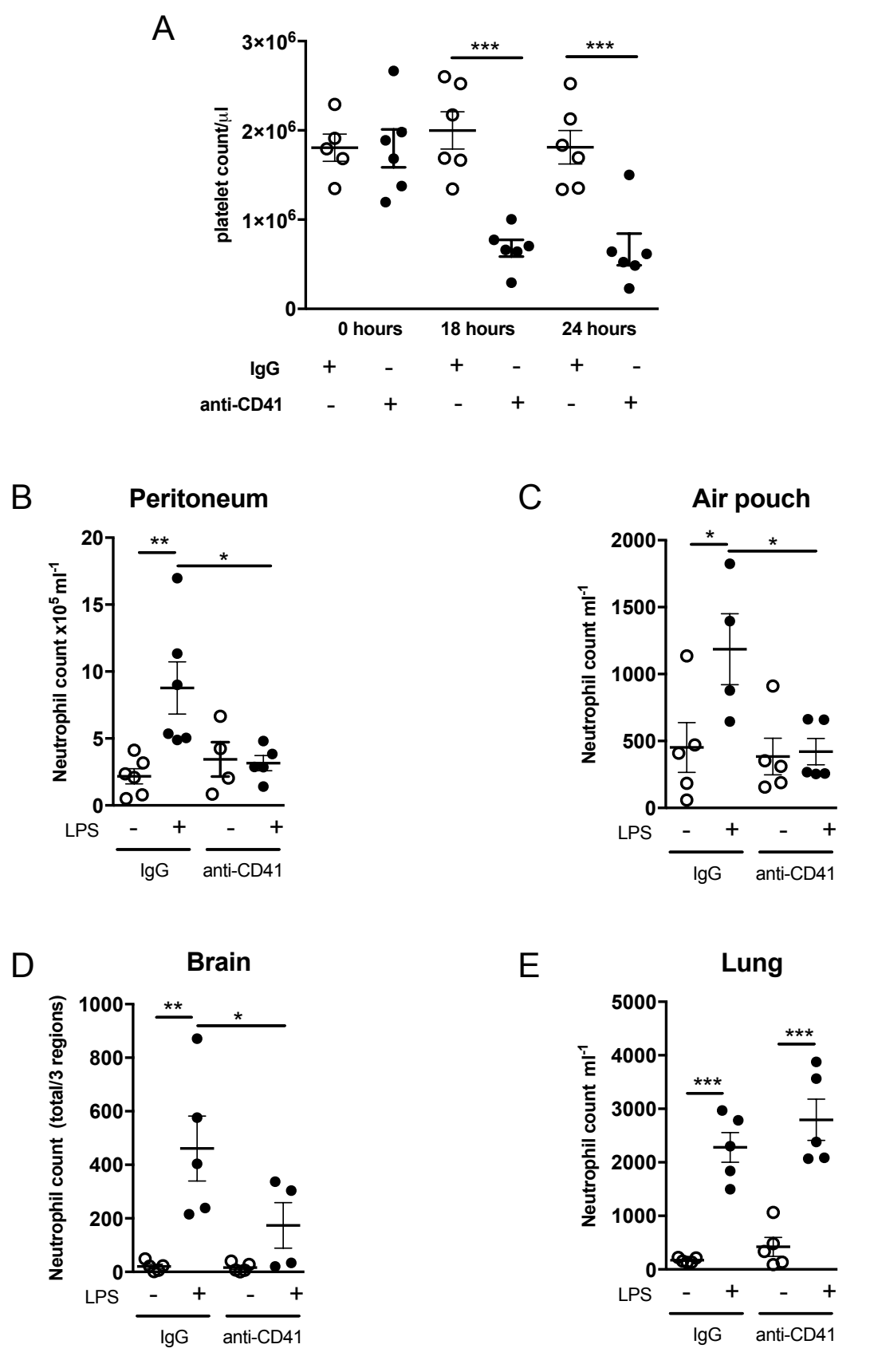
Figure 1. (A) Characterisation of platelet reduction via anti-CD41 antibody. Anti-CD41 antibody or IgG control were administered intraperitoneally. Blood was sampled via tail vein sampling at 0, 18 and 24h post-injection. Quantification of platelets was carried out via flow cytometry using BD TruCOUNT™ tubes. Individual data points are presented as a scatter graph with the mean ± SEM shown. ***p<0.001; one-way ANOVA with Bonferroni's correction. (B-E) Neutrophil infiltration to the peritoneum, skin and brain is platelet dependent after LPS-induced inflammation. The innate immune response was triggered by LPS challenge in four different tissues four hours after mice had received either anti-CD41 antibody or control IgG (1 mg/kg), and neutrophil accumulation was measured. Neutrophil infiltration is dependent on platelets during inflammation

in (B) the peritoneum, (C) subcutaneous air pouch and (D) Brain. Conversely, (E) neutrophil infiltration to the lung is not affected by platelet reduction after LPS injection. *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA with Bonferroni's correction. Individual data points are presented as a scatter graph with the mean ± SEM shown.

Figure 2. Chemokines CXCL1 and CCL5 in the inflamed lung are upregulated as compensatory mechanisms in response to platelet reduction. The cytokines IL-1α, IL-1β, IL-6, TNF and chemokines CXCL1 and CCL5 concentrations were measured in lavage fluid of peritoneum, air pouch and lung, or homogenised brain tissue after LPS challenge, in mice that had received either anti-CD41 antibody or control IgG. LPS induced a similar profile of inflammation across all tissue sites and was unaffected by platelet reduction in peritoneum, air pouch and brain. During inflammation, platelet reduction induced a significant increase in CXCL1 and CCL5 in lung lavage fluid. *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA with Bonferroni's correction. Individual data points are presented as a scatter graph with the mean ± SEM shown.

Figure 3. Non-depleting targeting of platelets reduces neutrophil infiltration to the brain. Anti-GpIbα antibody or IgG control were administered (4 mg/kg i.p) 4 hours before LPS-induced brain inflammation. Anti-GpIbα antibody had no effect on numbers of circulating platelets compared to IgG-injected controls (A) yet significantly reduced the number of neutrophils in brain tissue compared to IgG control during inflammation (B). Representative immunofluorescence

staining of reduced neutrophil numbers in the brain striatum following LPS injection in the presence of the anti-GpIbα antibody versus IgG control, which were not accompanied by any change in cerebrovascular activation (VCAM-1 statining) (C). **p<0.01, ***p<0.001; one-way ANOVA with Bonferroni's correction. Individual data points are presented as a scatter graph with the mean ± SEM shown. Scale bar = 200μm.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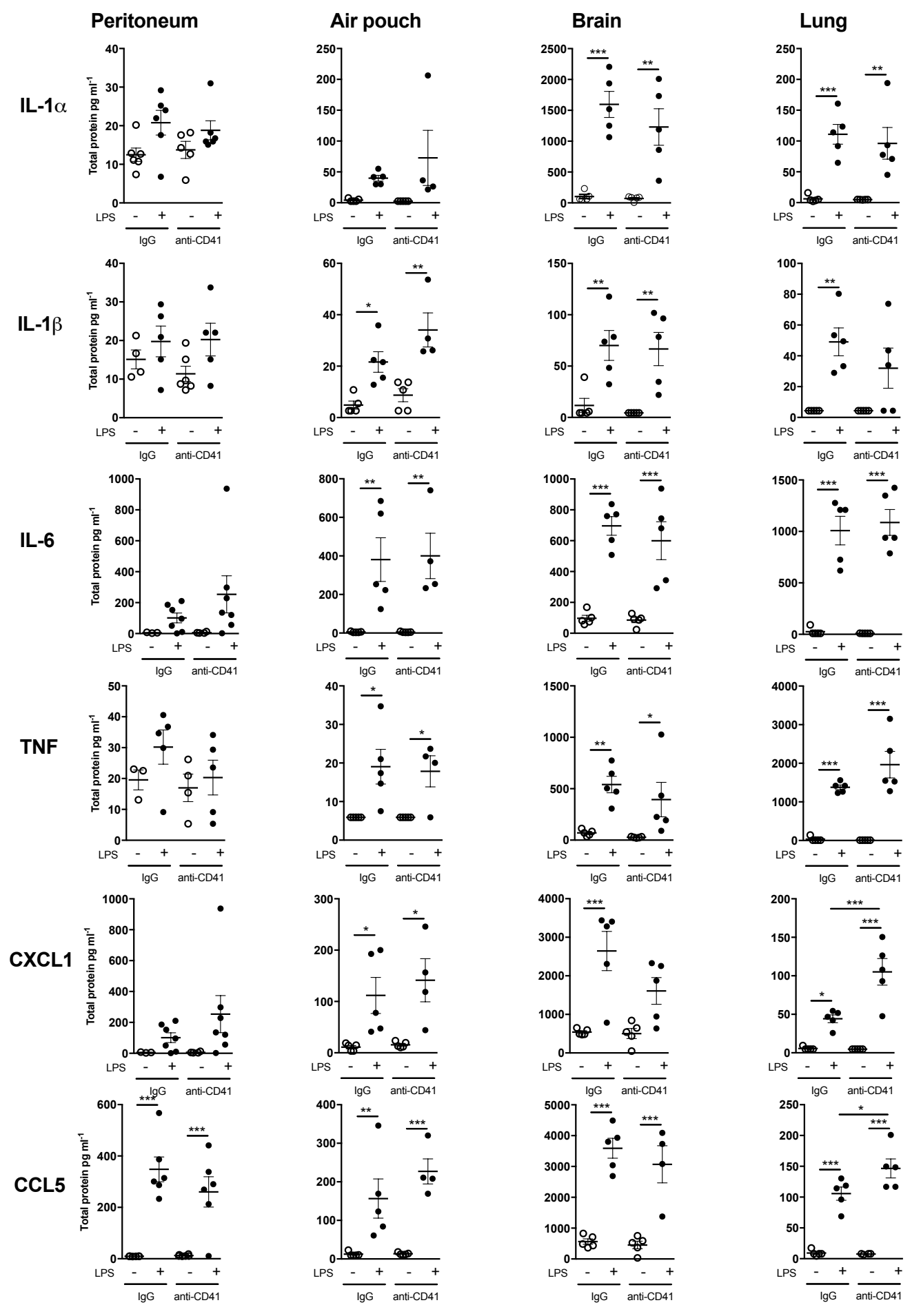
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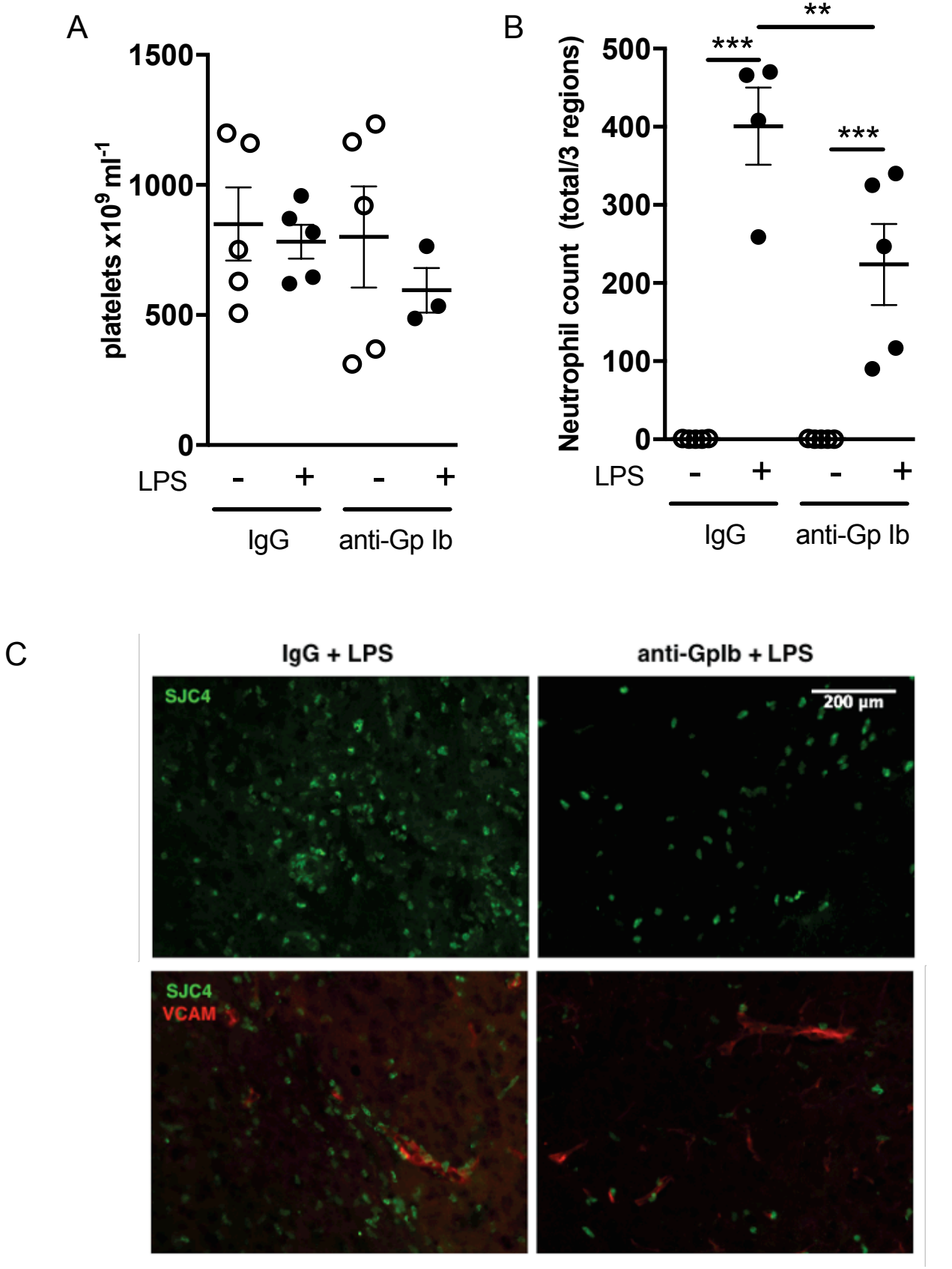
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