BRAIN COMMUNICATIONS

B lymphocyte responses in Parkinson's disease and their possible significance in disease progression

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Inflammation contributes to Parkinson's disease pathogenesis. We hypothesized that B lymphocytes are involved in Parkinson's disease progression. We measured antibodies to alpha-synuclein and tau in serum from patients with rapid eye movement sleep behaviour disorder ($n = 79$), early Parkinson's disease ($n = 50$) and matched controls ($n = 50$). Rapid eye movement sleep behaviour disorder cases were stratified by risk of progression to Parkinson's disease (low risk = 30, high risk = 49). We also measured B-cell activating factor of the tumour necrosis factor receptor family, C-reactive protein and total immunoglobulin G. We found elevated levels of antibodies to alpha-synuclein fibrils in rapid eye movement sleep behaviour disorder patients at high risk of Parkinson's disease conversion (ANOVA, $P < 0.001$) and lower S129D peptide-specific antibodies in those at low risk (ANOVA, $P < 0.001$). An early humoral response to alpha-synuclein is therefore detectable prior to the development of Parkinson's disease. Peripheral B lymphocyte phenotyping using flow cytometry in early Parkinson's disease patients and matched controls ($n = 41$ per group) revealed reduced B cells in Parkinson's disease, particularly in those at higher risk of developing an early dementia [$t(3) = 2.87$, $P = 0.01$]. Patients with a greater proportion of regulatory B cells had better motor scores [$F(4,24) = 3.612$, $P = 0.019$], suggesting they have a protective role in Parkinson's disease. In contrast, B cells isolated from Parkinson's disease patients at higher risk of dementia had greater cytokine (interleukin 6 and interleukin 10) responses following in vitro stimulation. We assessed peripheral blood lymphocytes in alpha-synuclein transgenic mouse models of Parkinson's disease: they also had reduced B cells, suggesting this is related to alpha-synuclein pathology. In a toxin-based mouse model of Parkinson's disease, B-cell deficiency or depletion resulted in worse pathological and behavioural outcomes, supporting the conclusion that B cells play an early protective role in dopaminergic cell loss. In conclusion, we found changes in the B-cell compartment associated with risk of disease progression in rapid eye movement sleep behaviour disorder (higher alpha-synuclein antibodies) and early Parkinson's disease (lower levels of B lymphocytes that were more reactive to stimulation). Regulatory B cells play a protective role in a mouse model, potentially by attenuating inflammation and dopaminergic cell loss. B cells are therefore likely to be involved in the pathogenesis of Parkinson's disease, albeit in a complex way, and thus warrant consideration as a therapeutic target.

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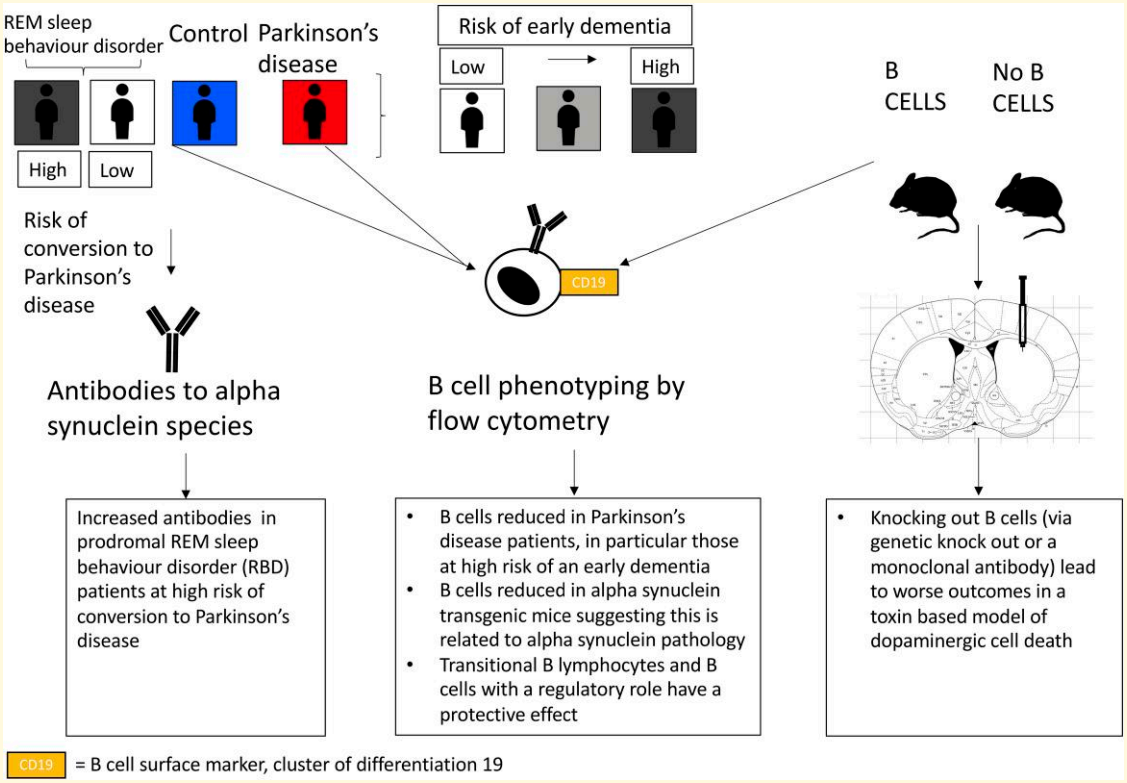
Abbreviations: ACE-R = Addenbrooke's Cognitive Examination Revised; BAFF = B-cell activating factor of the tumour necrosis factor receptor family; BSA = bovine serum albumin; CRP = C-reactive protein; CV = coefficient of variation; GM-CSF = granulocyte macrophage colony-stimulating factor; IL6 = interleukin 6; IL10 = interleukin 10; MAPT = microtubule-associated protein tau; MDS-UPDRS = Movement Disorder Society Unified Parkinson's Disease Rating Scale; MHC = major histocompatibility complex; MMSE = Mini-Mental State Examination; MSD = Mesoscale Discovery; NIHR = National Institutes of Health Research; 6-OHDA = 6-hydroxydopamine; PAMP = pathogen-associated molecular motif; PBMC = peripheral blood mononuclear cell; PBS = phosphate-buffered saline; PFA = paraformaldehyde; PMA = phorbol 12-myristate 13-acetate; RBD = REM sleep behaviour disorder; REM = rapid eye movement; RPM = revolutions per minute; SNCA = alpha-synuclein; TH = tyrosine hydroxylase; UPDRS = Unified Parkinson's Disease Rating Scale

Graphical Abstract



Introduction

Parkinson's disease is a common neurodegenerative disorder, affecting 2–3% of people over the age of 65.[1] The disease course is complicated by dementia in nearly half of patients within 10 years of diagnosis,[2] and there are no disease modifying treatments. Prior to developing overt Parkinson's disease, there is also a well-described prodromal phase consisting of non-motor features such as rapid eye movement (REM) sleep behaviour disorder (RBD), constipation and hyposmia, which may precede disease onset by a decade.[3,4] What determines whether, and at what rate, patients progress from this prodromal state to Parkinson's disease is unknown.

The central role of alpha-synuclein pathology is well-described in Parkinson's disease.[5-10] There is also evidence for an immune response to alpha-synuclein with the identification of T cells specific to alpha-synuclein in Parkinson's

disease patients[6] as well as alpha-synuclein–specific antibodies (reviewed in Scott *et al.*[7]). T-cell responses to alpha-synuclein were highest shortly after diagnosis of motor Parkinson's disease in larger cohorts,[8] suggesting that an adaptive immune response may peak close to disease onset. We have also shown that peripheral immune activation has prognostic significance in early Parkinson's disease.[9] Post-mortem studies have confirmed the presence of T-cell infiltrates in the brain in Parkinson's disease, with greater numbers in dementia cases and their presence correlated with microglial activation and alpha-synuclein aggregates in regions relevant to cognition.[10]

B lymphocytes not only produce antibodies via terminal differentiation into plasma cells but also have antibody-independent functions. They have immunoregulatory capacity, attenuating T-cell responses via the production of IL10 and IL35.[11-14] These so-called regulatory B cells are enriched in $CD24^{HI}CD38^{HI}$ transitional B cells[15] and in innate $CD1d^{+}CD5^{+}$ populations.[16] Furthermore, B cells can act as antigen presenting cells that activate CD4 T cells, produce pro-inflammatory cytokines [including granulocyte macrophage colony-stimulating factor (GM-CSF)[17] and interleukin 6 (IL6)[18]] and respond to pathogen-associated molecular motifs (PAMPS) via Toll-like receptor or B-cell receptor engagement.[19] B cells also comprise a quarter of meningeal immune cells in homoeostasis,[20] and more recently, IgA-producing plasma cells (typically found at mucosal surfaces) have been identified in the meningeal dural sinuses, with evidence that they are 'trained' in the gut and then home to the meninges to protect the brain.[21] Given the pleiotropic roles of B cells across the immune response, including CD4 T-cell activation and their presence in the meninges, we reasoned that they may be involved in the pathogenesis and progression of Parkinson's disease.

It is relatively well-established that B lymphocytes are reduced in Parkinson's disease,[22-25] although this is not replicated in all studies.[26] More recent studies have shown that alterations in B-cell subsets are also relevant with an increase in effector activity (increased cytokine production or memory B cells) in patients compared to controls[24,26] or a decrease in regulatory B cells.[27] Single-cell RNA sequencing of peripheral blood in a small sample (eight patients, six controls) showed clonal expansion of memory B cells with upregulation of major histocompatibility complex (MHC) Class II highlighting relevance of B cells as antigen presenting cells and their potential role in activating T cells.[28] It is unclear what role these changes have in disease progression.

We therefore sought to examine antibody responses to alpha-synuclein species in a prodromal RBD and early Parkinson's disease patient cohort together with well-matched controls, along with comprehensive phenotyping of peripheral B cells. We performed supportive mechanistic studies in relevant mouse models of Parkinson's disease.

Table 1 Participant clinical and demographic information (antibody study)

Variable	Control ($n = 50$)	Low-risk RBD ($n = 30$)	High-risk RBD ($n = 49$)	Parkinson's disease ($n = 50$)
Age	67.72 (5.09)	62.10 (9.52)[a]	67.72 (5.93)	68.01 (6.47)
Gender (% male)	55	80	87.8	66
Disease duration				3.2 (2.6)
MDS-UPDRS				34.3 (13.65)
MMSE				28.52 (2)
LEDD (mg)				517.37 (367.66)

MDS-UPDRS, Movement Disorders Society Unified Parkinson's Disease Rating Scale; MMSE, Mini-Mental State Examination; LEDD, levodopa equivalent daily dose; RBD, REM sleep behaviour disorder.

[a]Age was compared using a one-way ANOVA with *post hoc* *t*-tests. Low-risk RBD patients were significantly younger than the other groups ($P < 0.002$). The gender distribution was compared with chi-square tests. There was no significant difference between groups.

Materials and methods

The clinical studies were approved by the Cambridgeshire Research Ethics Committee (03/303) and South Central-Oxfordshire A Research Ethics Committee (ID 188167). Written informed consent was obtained from participants in accordance with the Declaration of Helsinki.

Study participants

Patients with idiopathic RBD were recruited through the Oxford Parkinson's Disease Centre (OPDC) Discovery Study from sleep disorder clinics at the John Radcliffe Hospital in Oxford, Papworth Hospital in Cambridge and Sheffield Teaching Hospital. RBD diagnosis, as described by Barber *et al.*,[29] was based on polysomnographic evidence according to the International Classification of Sleep Disorders criteria.[30] Idiopathic RBD patients were gender matched to Parkinson's disease patients and controls and stratified into low and high conversion risk prodromal Parkinson's disease groups (see Table 1). A probability score was calculated for prodromal Parkinson's disease for each REM sleep behaviour participant at their baseline assessment, based on the Movement Disorder Society (MDS) criteria.[31] A threshold of 80% was applied for stratification: patients with scores >80% were stratified into the 'high-risk' prodromal Parkinson's disease group, while those with scores lower than 80% were stratified into the 'low-risk' prodromal Parkinson's disease group. Cases who were current users of immunomodulatory therapies or with known autoimmune disorders were excluded.

Parkinson's disease cases were recruited from the Cambridge Parkinson's Disease Research Clinic. Inclusion criteria were fulfilment of the UK Parkinson's disease Brain Bank Criteria for a diagnosis of Parkinson's disease, age 55–80 years and early-stage disease (Hoehn and Yahr stage ≤ 2). Exclusion criteria included the presence of other neurodegenerative disorders, chronic inflammatory/autoimmune disorders, current infection, surgery in the last month, vaccinations in the previous 3 weeks or recent use of anti-inflammatory/immunomodulating

medications (steroids in the previous 3 months, high-dose aspirin >75 mg in the previous 2 weeks, ibuprofen and other non-steroidal anti-inflammatory drugs in the previous 2 weeks or any long-term immunosuppressants). Parkinson's disease cases were stratified into groups at high, intermediate and low risk of developing an early dementia using microtubule-associated protein tau (MAPT) genotype and neuropsychological predictors identified previously in a longitudinal cohort study of incident Parkinson's disease.[32] See Supplementary Methods for further details.

Immunophenotyping controls were recruited from the National Institutes of Health Research (NIHR) Cambridge BioResource (www.cambridgebioresource.group.cam.ac.uk) and were age, gender and MAPT genotype matched to Parkinson's disease patients. Further controls for the antibody study were recruited opportunistically from spouses of patients. Controls had no history of neurological disease, memory problems or depression. Exclusion criteria were the same as for the patients. See Supplementary Materials and Methods for details regarding blood sampling.

Antibody assays

Antibody levels to relevant antigens of interest were measured using custom Mesoscale Discovery (MSD) electrochemiluminescence assays in RBD, early Parkinson's disease patients and controls. Antigen preparation is described in full in the Supplementary Methods. All serum samples were run in duplicate. Streptavidin plates (MSD) were blocked with 150 µL of 3% bovine serum albumin (BSA) (Millipore, cat. no. 82–045-1) in phosphate-buffered saline (PBS) for 1 h on a shaker at 4000 revolutions per minute (RPM). They were washed three times with 150 µL 0.05% Tween 20 (Sigma-Aldrich P1279) PBS. The plates were then coated with 25 µL of antigen and incubated overnight at 4°C, then washed three times with 0.05% Tween 20 PBS. Calibrator antibodies were prepared as described in the Supplementary Methods. Serum was diluted 1:25 in 1% BSA in PBS. Diluted serum or calibrator was added in volumes of 25 µL. Plates were incubated for 1 h at room temperature on a shaker at 4000 RPM. They were washed again with 0.05% Tween 20 PBS three times. MSD sulfo-tag anti-human antibody was then added to the test wells and MSD sulfo-tag anti-mouse or anti-rabbit to the calibrator wells at a concentration of 1:500. The plates were then incubated at room temperature for an hour on a shaker at 4000 RPM. After incubation, plates were washed three times with 0.05% Tween 20 PBS and 150 µL of 1× read buffer was added. Electrochemiluminescence was read on an MSD plate reader (Sector S 600). Absolute concentrations were calculated from standard curves.

Serum IgG/CRP and BAFF measurements

The MSD human total IgG panel (K15203D) was used to measure total IgG, and the MSD V-PLEX human C-reactive protein (CRP) (K151STD) for CRP. B-cell activating factor of the tumour necrosis factor receptor family (BAFF) was measured using the human BAFF ELISA Kit (ab119579, Abcam) as per the manufacturer's instructions. Samples were run in duplicate.

PBMC isolation and immunophenotyping in Parkinson's disease patients and controls

Blood samples for peripheral blood mononuclear cell (PBMC) isolation from matched Parkinson's disease and control pairs were collected and processed on the same day and the same time (see Supplementary Materials and Methods for further details). PBMCs were isolated from heparinized blood using a Ficoll gradient within 2 h of phlebotomy. Isolated PBMCs were left to block in buffer for 10 min at 4°C (0.1% BSA, Probumin. Millipore, cat. no. 82-045-1; 0.01% sodium azide, Sigma-Aldrich, cat. no. S2002 made up in PBS; 2% mouse serum, Sigma-Aldrich, cat. no. M5905). Next, 0.5 µL of zombie aqua (Biolegend 423101) was added and incubated for 15 min prior to addition of surface staining antibodies (see Supplementary Methods). Samples were incubated at 4°C for 30 min. The PBMCs were then washed twice before being fixed in 2% paraformaldehyde (PFA) for 20 min. Flow cytometry was run within 2–4 h. Flow Cytometry Standard (FCS) output files were imported into FlowJo software (version 10.5.0), which was used for gating of the cell populations, which was done blinded to group. All flow cytometry was run on the BD Fortessa II flow cytometer with a minimum of 5000 B cells recorded.

B lymphocyte stimulation

PBMCs were defrosted (see Supplementary Methods) and cultured for 48 h in 200 µL of RPMI cell culture media with glutamine (Gibco 21875-034) in a 96-well round bottom plate. The test wells were stimulated with either CpG DNA (1:10 000) (Cambridge Bioscience #Hycult HC4039) and CD40 ligand (1:5000) (R and D systems, 6245-CL-050) or alpha-synuclein fibrils for 48 h. Five hours prior to removal from culture, phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) (Sigma-Aldrich #P8139), ionomycin (500 ng/mL) (Sigma-Aldrich #I0634) and brefeldin A (BFA) (5 µg/mL) (BioLegend #420601) (PIB) were added to the CD40L/CpG wells and to separate intermediate (PIB) wells. BFA was added to the alpha-synuclein and unstimulated wells.

Cells were removed from culture at 48 h and centrifuged at 350*g* for 5 min. Surface staining was done as per the protocol above excluding the fixation step. Cells were fixed and permeabilized prior to intracellular staining using the eBiosciences fixation and permeabilization kit as per manufacturer's instructions [Fix/Perm concentrate (00-5123-43) Fix/Perm Diluent (00-5223-56) and 10× Perm buffer (00-8333-56)]. See Supplementary Methods for a list of antibodies. Flow cytometry was run within 2–4 h.

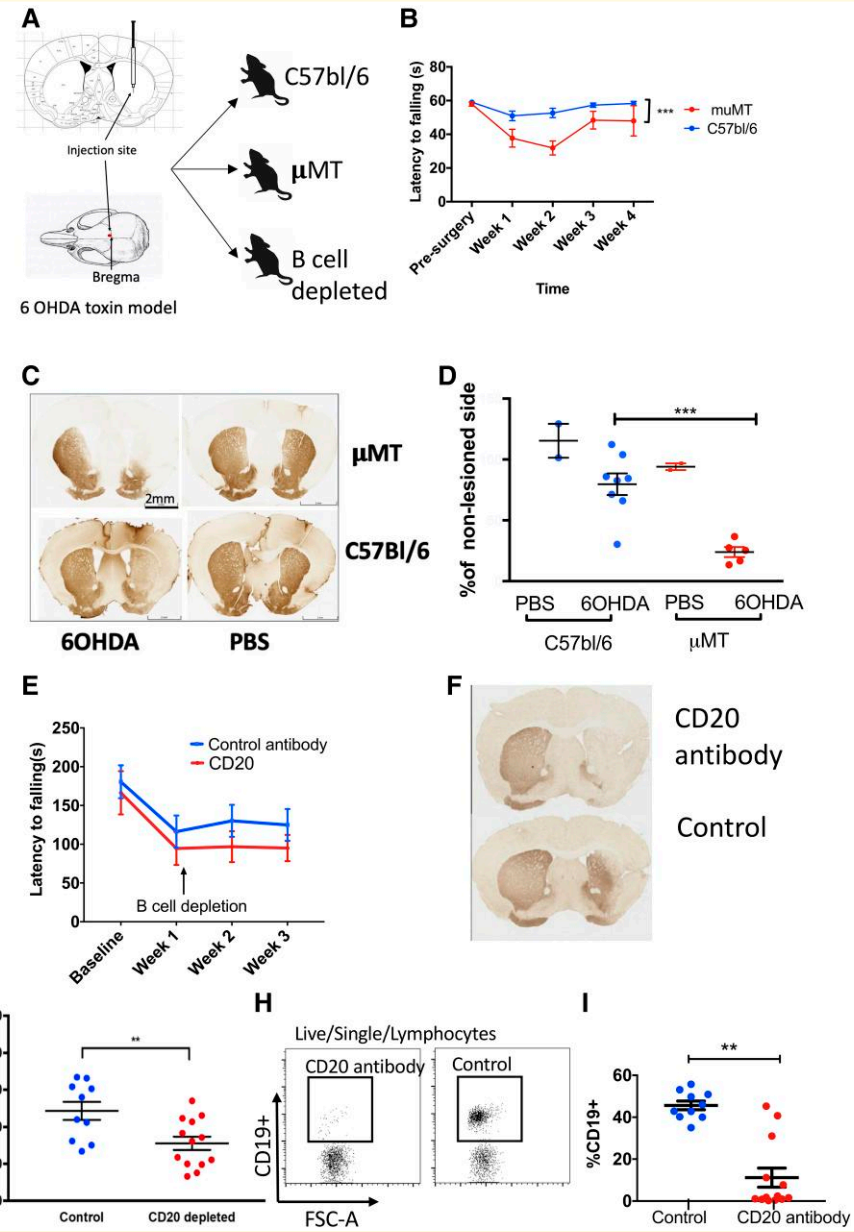


Figure 7 Pathological and motor outcomes are worse in B-cell–depleted 6-OHDA mouse model of Parkinson's disease. (**A**) The 6-OHDA toxin model showing the injection site in the striatum. (**B**) Motor performance on the rotarod test for $N = 28$ µMT mice (15 male) and $N = 28$ C57bl/6 mice (11 male) collected across two experiments (with equal numbers in each group for both experiments). All mice were aged between 11 and 14 weeks at the time of the experiment. The figure shows the mean and standard error of the mean (SEM). Lower latencies suggest poorer performance (mice fall off sooner). This short protocol test has a ceiling effect (a limit of 60 s on the bar) meaning that the C57bl/6 did not show a significant change after surgery. A two-way ANOVA was undertaken with genotype and week of testing as independent variables and showed that there was a significant main effect of genotype overall [$F(1,133) = 26.65$, $P < 0.0001$] and of time [$F(4,133) = 10.33$, $P < 0.0001$] with a significant interaction between the two terms [$F(4,133) = 3.02$, $P = 0.02$]. (**C**) Representative histological image showing TH^+ staining with a more extensive loss of dopaminergic fibres in the µMT mouse (examples from two individual mice: one µMT and one C57bl/6 mouse; both female, 12 weeks). (**D**) Quantification of the mean optical density of staining in the striatum (taken from 6–10 sections per mouse) showing the staining as a percentage of the non-lesioned side ($N = 8$ C57bl/6, $N = 5$ µMT) from mice culled 4 weeks after surgery. Each point represents one individual. Unpaired *t*-test, $t(11) = 4.07$, $P = 0.0006$. (**E**) Motor performance on the rotarod test for mice given CD20 antibody ($N = 10$) and control antibody ($N = 13$) female mice aged between 22 and 26 weeks. A two-way ANOVA with treatment and week of testing as independent variables showed there was a significant main effect of week of testing [$F(3,72) = 9.44$, $P = 0.0001$]. There was no main effect of treatment type [$F(1,24) = 1.05$, $P = 0.32$]. (**F**) Representative histological image showing TH^+ staining with more extensive loss of dopaminergic fibres in a mouse given a CD20 antibody versus a mouse given the control antibody (examples from two individual mice). (**G**) Quantification of the mean optical density of staining (across 6–10 sections for each mouse) in female C57/bl6 mice (12 weeks old) given either control ($N = 10$) or an anti-CD20 monoclonal antibody ($N = 13$). Unpaired *t*-test, $P = 0.01$. (**H**) Representative flow cytometry gating plot showing B cell ($CD19^+$) gating in a mouse given the control antibody and a mouse given the CD20 antibody (peripheral blood). Each point represents a single cell. (**I**) B cell percentages in individual mice calculated from flow cytometry plots in the CD20 and control groups. Unpaired *t*-test, $t(21) = 6.22$, $P = 0.001$. TH, tyrosine hydroxylase.

is required to show a more nuanced antibody signature (as suggested in Folke *et al*.,[41] who describe a complex phenotype in multiple system atrophy versus Parkinson's disease versus controls). Importantly, our study did find a difference compared to healthy controls, but at a much earlier time point in the disease, namely in the prodromal phase, consistent with the overall hypothesis that antibody responses are increased in early disease but decrease as the disease progresses.

Therapeutic trials of alpha-synuclein antibodies in Parkinson's disease are underway[42] with initial data showing a decrease in blood and CSF alpha-synuclein levels. Antibodies may have beneficial effects, mediating the clearance of some pathological forms of alpha-synuclein (for example via Fcγ receptor-mediated phagocytosis). Alternatively, antibodies could contribute to disease progression by promoting an inflammatory milieu in the brain (e.g. via activation of the complement cascade), leading to microglial activation, peripheral immune cell recruitment and further neuronal death.

We also investigated peripheral blood B-cell number and phenotype in patients with established Parkinson's disease. In line with previous studies, we found a decrease in total B-cell number compared with controls,[22,23,25] but here we describe (for the first time) that this observation is driven by a marked decrease in B cells in the patient subset at highest risk of developing an early dementia. The B cells remaining in these high-risk patients also showed a greater cytokine response (both IL10 and IL6) when stimulated *ex vivo* compared with controls consistent with recent studies[26] and also had higher expression of the activation marker CD25, consistent with *in vivo* priming. Thus, in this subgroup of patients, the B cells are both depleted in number but more activated, suggesting that they may play a role in driving pathology in those with a worse prognosis. The decrease in circulating B lymphocyte numbers was not clearly driven by a decrease in any one subset (rather appearing to be a global reduction in B cells). This finding was recapitulated in two alpha-synuclein transgenic mouse models of Parkinson's disease, suggesting that this is associated with a response to pathological alpha-synuclein rather than other parameters relating to disease in humans.

All of this data would therefore suggest that that B cells are activated in Parkinson's disease, particularly in those at highest risk of disease progression and that this is associated with a reduction in circulating B cells, either due to their exit from the blood (potentially to the CNS or gut, where alpha-synuclein pathology is well-described[43]) or due to activation-induced cell death. In one recent study, Yan *et al*.[26] found a decrease in $CXCR3^+$ B cells that may reflect homing to lymphoid tissue or the menginges by these cells. Further work is required to delineate between these possible explanations.

Our analysis of B-cell subsets also generated some interesting observations: Parkinson's disease patients in our cohort had fewer CD1d positive cells than their matched controls. We also noted that a higher proportion of circulating $CD24^{HI}CD38^{HI}$ transitional B cell and other subsets enriched for 'regulatory' IL10-producing B cells, including CD1d and $CD5^+$ cells,[16] was associated with better motor scores (lower MDS-UPDRS-III scores). Interestingly, in mice, CD1d-mediated presentation of lipid antigens by regulatory B cells to invariant NKT cells resulted in suppressive iNKT capable of modulating pathological autoreactive Th17 and Th1 CD4 T cells.[44] Our data therefore suggest that B cells with regulatory capacity may be beneficial in Parkinson's disease, although whether that requires CD1d or iNKT activation by B cells or is due to direct effects on pathological T cell responses via IL10 production will need further study. Regardless of the mechanism, our observations are consistent with the previously described protective effects of T regulatory cells in Parkinson's disease (reviewed in Chen *et al*.[45]) and the more recently described decrease in functional regulatory B cells (as characterized by surface markers and *in vitro* effector function) in Parkinson's disease compared to controls.[27] This suggests that boosting regulatory T or B lymphocytes may be a useful therapeutic strategy to prevent disease progression. We would argue that it is the balance between the effector and regulatory subsets that results in a protective or deleterious effect.

In this respect, the study we undertook using a toxin-based mouse model supported the suggestion from our human immunophenotyping data that some B-cell subsets may play a protective role in Parkinson's disease. We found that B-cell deficiency led to worse behavioural outcomes. B-cell deficiency or antibody-based depletion was associated with a larger dopaminergic cell loss. Previous studies have examined the effect of lymphocyte deficiency on motor and histological phenotypes in toxin-based models of Parkinson's disease, one using athymic rats[46] and another using $RAG^{-/-}$ mice that lack both T and B lymphocytes.[47] Both of these studies noted worse outcomes in lymphocyte-deficient mice rescued by bone marrow transplantation.[47] Depleting $CD4^+$ T cells alone appears to improve outcomes in animal models.[48] Taken together with our results, one could hypothesize that the rescue obtained with bone marrow transplantation in lymphocyte-deficient mice is at least in part mediated by the protective effects of regulatory B cells. Further work examining the effect of transferring B lymphocytes enriched for a regulatory phenotype is required. We also acknowledge that an isotype control would be a more appropriate control for the antibody depletion than the chimeric version of the CD20 antibody (rituximab). Human IgG Fc from the constant portion of the antibody has excellent binding to mouse Fcγ receptors therefore is still a useful control for non-specific Fc-dependent antibody effects. The overall protective effect of B cells at least in this model shows that they have an action that is independent of alpha-synuclein pathology and related in some way to cell death and/or microglial activation.

The strengths of this study include the provision of well-matched age, gender and genotype controls for both a prodromal RBD and early Parkinson's disease patient cohort stratified by risk of disease progression as well as the intensity and depth of analysis we have undertaken looking at different types of B cells and their function. Previous studies have been limited by the heterogeneity of clinical cohorts and sub-optimal control groups.[7] We have also focussed on identifying immunological factors associated with clinical

progression rather than diagnosis, namely RBD to Parkinson's disease conversion and risk of progression to an early dementia in established Parkinson's disease, utilizing our well-validated markers to identify patients at risk of worse outcomes and showing that changes in the B-cell compartment are related to risk of disease progression. Potential confounding factors in our study include the effects of dopaminergic medication on immune cells, as most immune cells express dopamine receptors.[49] The study is also limited by the relatively small sample number (although similar to published literature) and the lack of longitudinal follow up. In future work, we would like to explore B-cell phenotypes in prodromal Parkinson's disease cases, to allow us to determine how early in the disease the observed functional B-cell changes occur, and to explore the relationship between antibodies and B-cell phenotypes in more detail. However, in our current study, we only had access to stored serum samples in the REM sleep behaviour cohort.

Overall, our results highlight that changes in the B-cell compartment are associated with disease progression and raise the possibility that therapeutic strategies augmenting regulatory B cells may have utility in very early Parkinson's disease or RBD.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

Data availability

Data are available on request.

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