Fisher Center for Alzheimer's Research Foundation

Scientific report

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General Introduction

AD is a devastating age-related neurodegenerative disorder, and the most frequent cause of senile dementia(1). The appearance of cognitive decline is associated with accumulation of misfolded proteins, as well as the presence of several additional toxic processes (2). Among the common neuropathological features found in AD are synaptic and neuronal loss, intracellular neurofibrillary tangles, elevated levels of the toxic form of amyloid beta (Aβ) (1–42), and the accumulation of extracellular senile plaques containing misfolded Aβ peptide (2-4). Local inflammatory responses as well as overwhelming astrocyte reactivity are often observed in the brains of AD patients and in rodent models; these processes are not necessarily the primary causes of the disease, but are considered to be key factors in disease progression and escalation (5-7). The accumulated misfolded proteins and the neuroinflammatory component have led to numerous attempts over the years to arrest disease progression, either using treatments that are directed against the misfolded proteins to arrest plaque burden (8, 9), or using systemic anti-inflammatory drugs to arrest the brain inflammation. Inconsistent and even conflicting results were reported, and none of the drugs tested thus far have proven effective in reversing or arresting cognitive loss in patients (10-16).

The failure of treatments directed at Aβ to arrest cognitive loss or to reverse it could reflect the fact that by the time Aβ plaque burden is high, removal of plaques, while still important, may be insufficient to modify disease because of numerous collateral disease-escalating factors that enter into a vicious cycle, which continues even after the plaques are removed. Such factors might include those whose mitigation is dependent directly or indirectly on the immune system. In apparent support of such a model are the results suggesting that resolution of inflammation requires an active mechanism mediated by circulating immune cell recruitment to sites of brain pathology (17-19).

Systemic leukocytes are essential players in CNS repair

For decades, it was commonly believed that the brain, and the CNS in general, is unable to tolerate immune cell entry, mainly due to the understanding that the brain is a tissue behind barriers, and is viewed as an immune privileged site (20). In animal models of acute CNS injuries, both monocyte-derived macrophages and $CD4^+$ T cells recognizing brain antigens, are needed for coping with and helping heal parenchymal damage (21-28). Moreover, T cells present in the periphery facilitate recruitment of the monocyte-derived macrophages to the CNS, and such macrophages play a role in supporting neuronal survival and facilitating axonal growth by resolving the local inflammatory response through their production of IL-10, and degradation of the local scar by metalloproteinase secretion (25-27, 29-32). Additional studies revealed that systemic T cells not only participate in CNS repair, but are also needed for life-long brain plasticity (33-35).
In investigating how T cells support healthy brain plasticity while they are excluded from the brain parenchyma, how they facilitate recruitment of monocyte-derived macrophages, and how such

monocytes can gain access to the CNS without breaching the blood-brain-barrier (BBB), it was demonstrated that the brain's barriers, including the meningeal barrier (36, 37) and the epithelial cell layer (CP) within the BCSFB, can serve as key compartments for immune-brain crosstalk in health and disease (19, 38, 39). This finding, coupled with unique epithelial composition of the BCSFB relative to other CNS barriers, comprised of endothelial tight junctions (40-43), and the accumulated evidence that immune cells are needed for brain maintenance and repair, led us to discover that the blood-CSF-barrier is a physiological restrictive gate that enables selective immune cell access, depending on the needs of the CNS (19, 38).

The paradoxical fate of the "leukocyte gate" to the brain in Alzheimer's disease models

Several independent studies have shown that recruitment of circulating monocyte-derived macrophages (44-52), possibly together with that of additional immunoregulatory leukocytes, can modify AD pathology (31, 53, 54). Such cells can help remove misfolded protein such as Aβ-plaques (48, 55, 56), balance the local inflammatory milieu (46, 47, 57), reduce gliosis (58), and protect synaptic structures (46, 57, 59).

Analyzing the fate of the CP with respect to its ability to support leukocyte trafficking, it became clear that its activity is impaired in brain aging and in animal models of AD (60, 61). It was further discovered that transiently reducing systemic immune suppression in AD animal models, by depleting peripheral $Foxp3^+$ regulatory T cells, augments IFN-γ activity in the circulation as well as its availability at the CP, and has a beneficial effect in mitigating disease pathology (62). These results are consistent with an independent observation, showing that the adaptive immune system plays an important role in AD etiology; it was demonstrated that genetic ablation of B, T, and natural killer cells in the 5XFAD mouse model by crossing these mice with Rag2/Il2rc double knockout animals (Rag-5xFAD), results in increased plaque load and increased soluble Aβ levels (63).

Importantly, although immunoregulatory and anti-inflammatory cells are needed in the brain parenchyma as a source of anti-inflammatory cytokines for reducing the inflammatory response, their homing to the brain requires well-controlled boosting of systemic immunity, to enable opening of the gateway to the CNS. Therefore, special care must be taken when viewing immunosuppressive cells (such as FoxP3) as uniformly beneficial or harmful in neurodegenerative diseases, without regard to their localization and kinetics.

Taken together, the results summarized above created the basis for our approach of empowering the systemic immune system, by transiently blocking inhibitory immune checkpoints, to thereby drive a cascade of immunological events that start outside the brain, induce activation of the CP, and culminate in immune-dependent brain repair processes (61, 64).

Immune checkpoint blockade for mitigating AD pathology

Inhibitory immune checkpoints restrain the activity of memory T cells, mainly those directed against self-compounds, to avoid autoimmune diseases. Among such checkpoints are the Programmed cell death protein 1 (PD-1), a member of the B7-CD28 family, expressed by a variety of activated effector memory immune cells, including CD4+ T cells (65). The PD-L1 ligand is expressed by dendritic cells and regulatory T cells (66), as well as by non-immune cells such as endothelial and epithelial cells (67, 68), and astrocytes (66). The interaction between PD-1 and its PD-L1 ligand suppresses memory T-cell responses, including proliferation, and cytokine production (65, 69). Blocking the PD-L1/PD-1 pathway potentially results in an increase in T cell activation (70-72). Based on our new understanding that boosting of systemic immunity in a well-controlled manner can help fight against AD, we envisioned that targeting PD-1/PD-L1 might be an effective means to achieve such immune activation. Our studies using anti-PD-1 or PD-L1 antibody in the 5XFAD mouse model of AD as well as in a dementia model of tau pathology revealed that such treatments are effective in boosting levels of IFN-γ producing T cells, with a

consequent dramatic effect in mitigating cognitive decline and disease pathology. This process was associated with recruitment to the brain parenchyma of monocyte-derived macrophages (61, 62). Such monocytes locally express numerous molecules that can act as scavenger receptors for removal of misfolded or aggregated protein, promote an anti-inflammatory effect, and serve as a source of growth factors (61, 64). Importantly, while the effect on brain pathology was extremely robust, it did not require continuous administration of the treatment; thus, a single injection of antibody initiated a chain of events that started outside the brain and led to alterations in several processes within the brain that together resulted in disease mitigation. It takes approximately 1 month from the initial administration of antibody for such effects to be manifested (64).

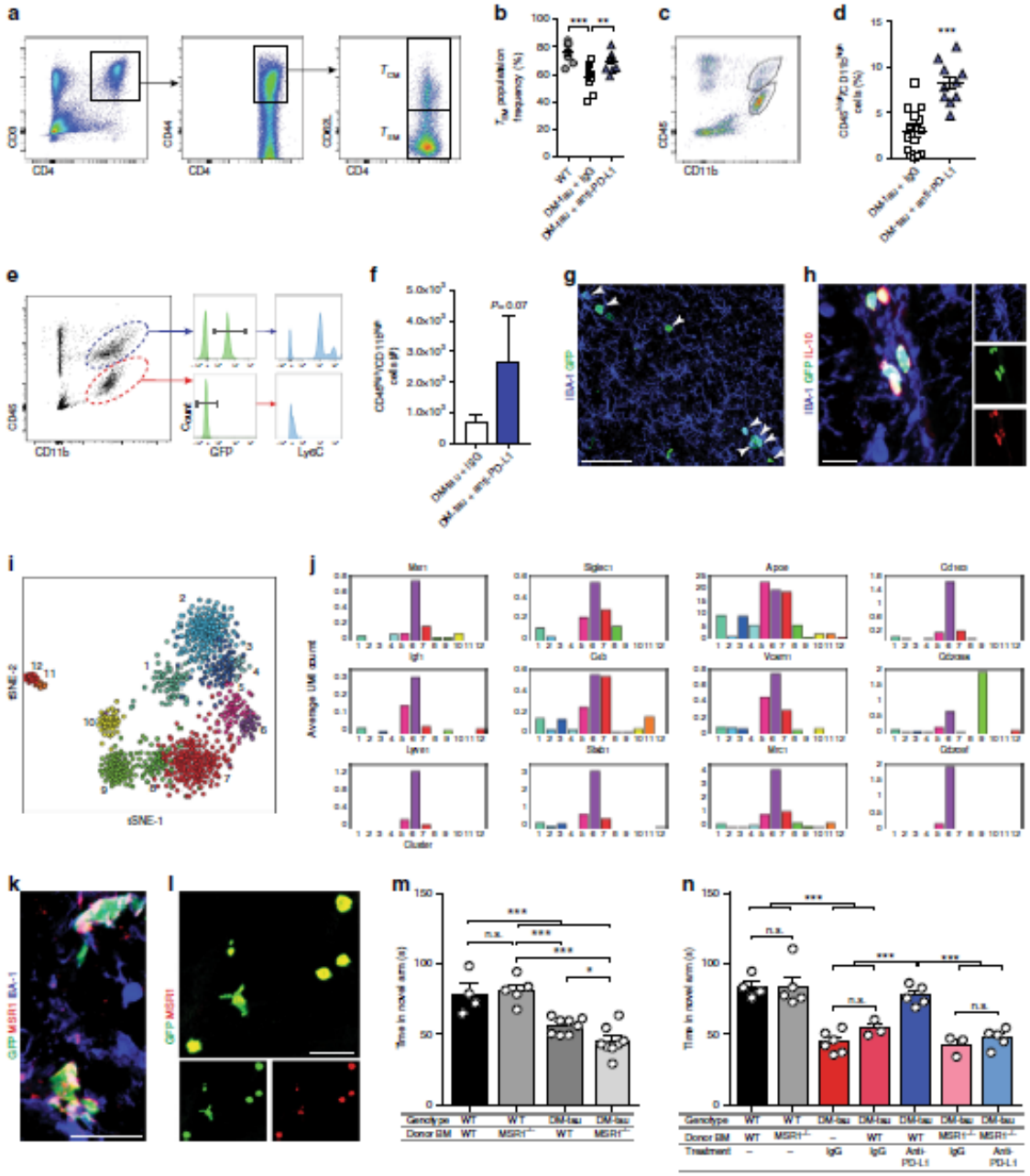
In this report, we describe our studies regarding the mechanism by which **targeting the PD-1/PD-L1 pathway in a mouse model of tau pathology enhances recruitment of monocyte-derived macrophages to the brain parenchyma, and present the phenotypic characterization of the recruited cells**.

In both 5XFAD and J20 mouse models of AD, disease progression is associated with a reduction of CP expression of leukocyte-trafficking molecules (73, 74). Treatment of 5XFAD mice with anti-PD-1 antibodies results in enhanced recruitment of monocyte-derived macrophages to the brain (61). These findings, together with our current results demonstrating a beneficial effect of anti-PD-L1 in the DM-hTAU model of dementia (75), prompted us to test whether the observed beneficial effect of targeting PD-L1 on cognitive function and disease pathology in this tau mouse model was associated with enhanced trafficking of immune cells to the diseased brain. To this end, we first tested whether the administration of antibody directed against PD-L1 induced elevation of effector memory T cells in DM-hTAU mice. Analyzing the spleens of DM-hTAU mice 2 weeks after anti-PD-L1 antibody administration revealed increased levels of effector memory T cells (T_{EM}; $CD44^{+}CD62L^{low}$) relative to those in IgG-treated mice (Fig. 1a, b), as evaluated by flow cytometry analysis. We further analyzed, by flow cytometry in the DM-hTAU mice, whether the treatment facilitated recruitment of monocyte-derived macrophages ($CD45^{high}CD11b^{high}$) to the brain parenchyma. We found a significant increase in $CD45^{high}CD11b^{high}$cells in the brains of DM-hTAU mice treated with anti-PD-L1 antibody relative to those treated with the IgG2b isotype control (Fig. 1c, d). To confirm the lineage of these cells, which we classified as mainly monocyte-derived macrophages based on their high expression of CD45 and CD11b, we repeated this experiment with bone marrow (BM)-chimeric mice, in which the donor BM cells were taken from mice with GFP-labeled hematopoietic cells (76). To create such chimera, recipient DM-hTAU mice were conditioned with lethal-dose irradiation, with the radiation beam targeting the lower part of the body while avoiding the head, prior to BM transplantation (25). Following establishment of chimerism, animals were treated with either anti-PD-L1 antibody or with control IgG2b. Analysis of the brains 2 weeks after the administration of the antibody, by flow cytometry, revealed that among the $CD45^{high}CD11b^{high}$ cells, about 50% of the cells were GFP^{+}, which was consistent with the extent of the chimerism, and confirmed their identity as infiltrating monocytes, rather than activated resident microglia (Fig. 1e, f). No GFP^{+} cells were seen among the $CD45^{low}CD11b^{+}$ cells. Notably, we gated only on $GFP^{+}CD45^{+}CD11b^{+}$ myeloid cells; BM-derived cells that were $GFP^{+}CD45^{+}CD11b^{-}$ were not analyzed. Treatment with anti-PD-L1 antibody resulted in an approximately threefold increase in the frequency of $GFP^{+}CD45^{high}CD11b^{high}$ cells, relative to IgG2b-treated control (Fig. 1f). Notably, this number underestimates the number of homing macrophages, since the chimerism was only about 50%. The brains from other mice from the same experiment were excised and processed for immunohistochemistry, which revealed the presence of $GFP^{+}IBA\text{-}1^{+}$ myeloid cells in the cortex of the anti-PD-L1-treated mice (Fig. 1g). We also stained brain sections from the same animals for the anti-inflammatory cytokine, IL-10, and observed its co-localization with infiltrating monocyte-derived macrophages, but not with $IBA\text{-}1^{+}GFP^{-}$ microglia (Fig. 1h).

The overall number of monocyte-derived macrophages that infiltrated the brain was low, and the number of those that were GFP^+ was even lower. Therefore, we further characterized the infiltrating cells by single-cell RNA-seq. We sorted all the $CD45^{high}CD11b^{high}$ cells from both IgG2b-treated and anti-PD-L1-treated groups, thereby enriching the monocyte-derived macrophages within the analyzed samples. Clustering analysis of 899 cells revealed that the infiltrating monocyte-derived macrophages were heterogeneous, and most likely included several activation states (as seen in clusters 5–10); clusters 1–4 represent activated microglia in several activation states, and clusters 11–12 indicate neutrophils. Analysis of differential genes in each cluster highlighted a unique signature displayed mainly by clusters 5 and 6, distinct from the resident homeostatic or activated microglia (clusters 1–4); the unique signature was manifested by expression of several molecules that could potentially mediate an important function in disease modification (Fig. 1i, j). One such uniquely expressed molecule is the macrophage scavenger receptor 1 (*Msr1*) (also known as SRA1, SCARA1, or CD204), an important phagocytic receptor required for engulfment of misfolded and aggregated proteins (77, 78), and found previously by us to be expressed by M2-like infiltrating monocyte-derived macrophages that are needed for spinal cord repair (38). Notably, these macrophages expressed additional relevant functional molecules, among which are the insulin-like growth factor-1 (*igf1*) that was previously reported to enhance neurogenesis in the aged brain (79), lymphatic endothelium-specific hyaluronan receptor (*lyve1*) and the scavenger receptor stabilin-1 (*Stab-1*) (Fig. 1j), both of which are markers of anti-inflammatory macrophages, associated with wound healing and lymphogenesis (80). Additional genes, found here to be uniquely expressed by infiltrating monocyte-derived macrophages, are scavenger receptors such as the sialic acid binding Ig-like lectin 1 (*Siglec1*) and the mannose receptor C-type (*Mrc1*) (Fig. 1j).

In light of the reported role of MSR1 in neurodegenerative diseases, we further focused on this scavenger receptor. Using immunohistochemistry, we confirmed the expression of MSR1 by the GFP^+ (infiltrating) cells (Fig. 1k, l), in line with our previous findings (61). Finally, to gain insight into the functional impact of MSR1-expressing macrophages on the repair process, we created BM chimeric DM-hTAU mice, in which the BM of the recipient mice was replaced with donor BM taken from MSR1-deficient mice. As controls we used DM-hTAU chimeric mice in which the recipient BM was replaced by BM taken from non-transgene wild-type littermates. Two weeks following BM transfer, the mice were examined for cognitive performance using the T-maze task. We also tested WT chimeric mice that received either wild-type BM or BM from $MSR1^{-/-}$ mice (Fig. 1m, n). Following the behavioral test, each group of DM-hTAU chimeric mice was divided into two groups that received either anti-PD-L1 antibody or the control IgG2b, and 4 weeks later were tested again for their performance in the T-maze. Another group of non-chimeric DM-hTAU littermates that received IgG2b control was evaluated in parallel. Anti-PD-L1 antibody reversed cognitive loss in DM-hTAU chimeras harboring BM from wild-type mice, while DM-hTAU chimeras harboring $MSR1^{-/-}$ BM lost the ability to respond to PD-L1 blocking antibody and failed to show improved cognitive ability (Fig. 1n).

Taken together, our results suggest that systemic immune activation, under conditions of chronic neuroinflammation, associated with murine models of tauopathies, facilitates the homing of monocyte-derived macrophages to the diseased brain and that these cells are key players in the anti-PD-L1 effect on disease modification.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WT
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DM-tau + anti-PD-L1
c
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DM-tau + IgG
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Figure 1: Monocyte-derived macrophages uniquely affect disease modification in PD-L1 blockade in DM-hTAU mice. **a, b** Flow cytometry of splenocytes, $CD44^{+}CD62L^{low}$ effector memory T (TEM) cells, versus $CD44^{+}CD62L^{high}$ central memory T (TCM) cells in DM-hTAU mice, treated with 0.5 mg of anti- PD-L1 (n = 10) or IgG (n =11) (one-way ANOVA, Fisher's exact test). **c, d** Flow cytometry of brains from anti-PD-L1-treated mice (n =10), and IgG-treated mice (n = 16) analyzed for $CD45^{high}CD11b^{high}$, pooled from two experiments. **e–g** Repeated experiment as in **a, b** using GFP-BM-chimeric DM-hTAU mice. e Flow cytometry of GFP-labeled cells gated from $CD45^{high}CD11b^{high}$ cells, expressing Ly6C. **f** Quantitation of the number of $GFP^{+}CD45^{high}CD11b^{high}$ cells in anti-PD-L1 (n = 4), relative to IgG-treated mice (n = 6). **g** Representative projections of confocal z-axis stacks, showing colocalization of GFP^{+} cells (green) with IBA-1 (blue), in the cortex of DM-$hTAU^{GFP/+}$ mice, treated with anti-PD-L1 antibody (arrowheads). Scale bar: 100 μm. **h** Representative confocal z-axis stacks, showing colocalization of GFP+ cells (green), IBA-1 (blue), and IL-10 (red) in the brains of anti-PD-L1-treated DM-hTAUGFP/+ mice. Scale bar: 50 μm. **i** Sorted $CD45^{high}CD11b^{high}$ from DM-hTAU mice treated with anti-PD-L1, analyzed by single-cell RNASeq. tSNE plot depicting 899 cells. Clusters indicated by color and number. **j** Average Unique Molecular Identifier counts for selected genes across the 12 clusters. **k, l** Representative projections of confocal z-axis stacks, showing colocalization of GFP+ cells (green) with MSR1 (red) and IBA-1 (blue) in the cortex (**k**), and of GFP+ cells (green) with MSR1 (red) in the hippocampus of DM-$hTAU^{GFP/+}$ mice treated with anti-PD-L1 antibody (**l**). Scale bars: 25 and 50 μm. **m, n** BM-chimeric DM-hTAU and WT mice (male and female) prepared using WT or $MSR1^{-/-}$ mice as BM donors. **m** T-maze task, 2 weeks after BM transplant, of WT >WT (n = 4), $MSR1^{-/-}$>WT (n =5), WT> DM-hTAU (n = 8) and $MSR1^{-/-}$ > DM-hTAU (n = 8) chimeric mice. **n** The same mice were treated after the behavioral assessment in m with 1.5 mg of anti-PD-L1 antibody or IgG control antibody, and were tested again 1 month later for their performance in T-maze; nonchimeric IgG-treated DM-hTAU littermates were used as additional controls. Improved performance of WT > DM-hTAU treated with anti-PD-L1 (n =5) versus IgG-treated WT > DM-hTAU (n = 3) and IgG-treated nonchimeric DM-hTAU mice (n= 6). $MSR1^{-/-}$ > DM-hTAU mice failed to show beneficial effect following treatment with anti-PD-L1 (n = 5), performing similarly to $MSR1^{-/-}$ > DM-hTAU treated with IgG (n = 3). In all panels, error bars represent mean ± s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA and Fisher's exact test)

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