2021

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative movement disorder in which symptoms derive from deficits in dopamine neurotransmitter levels secondary to loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) associated with misfolding and accumulation of α-synuclein. Neuroinflammation via microglia and T effector cells (Teffs) contribute to dopaminergic neuronal cell death. Recognition of cytokine profiles of pro-inflammatory microglia is not well understood and serve as potential therapeutic targets to reduce neuroinflammation. Recent studies demonstrated a novel Th17.1 Teff clonotype increases neurotoxicity. The aim of this study was to demonstrate *in vitro* cytokine responses by BV-2 microglia induced by Th1, Th17, and Th17.1 clonotypes to assess neuroinflammation mechanisms in PD. Cytokine responses by BV-2 microglia co-cultured with activated Teff clonotypes were analyzed using a cytokine membrane array. Co-culture with Teffs led to significant increases in the majority of cytokine responses from BV-2 microglia compared to control. Cross group analysis relative expression demonstrated variation in cytokine profiles produced between microglia treated with different Teff clonotypes, especially with regard to IFNγ, MIG, MIP-1α, TIMP-1, RANTES, SDF-1, and IL-12 p40/p70. Ingenuity Pathway Analysis (IPA) of cytokines displaying significant relative expression levels for each Teff clonotype showed Th1- and Th17- treated BV-2 microglia demonstrated pathways related to cellular movement, hematological development and function, and immune trafficking while Th17.1-treated microglia upregulated pathways related to disorders of connective tissue, inflammation, and organismal injury. In conclusion, Th1, Th17, and Th17.1 Teffs treatment of BV-2 microglia led to upregulation of most pro-inflammatory cytokines and pathways. However, specific Teff clonotype culture with BV-2 microglia displayed differing cytokine profile responses through varying relative expression profiles with significant differences related to IFNγ, MIG, MIP-1α, TIMP-1, RANTES, SDF-1, and IL-12 p40/p70 delineating alternative inflammatory pathways. These results provide relevant targets for strategies to attenuate neuroinflammation and protect dopaminergic neurons in PD

BACKGROUND

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Characteristic PD symptoms are due to the deficiency of dopamine neurotransmitter resulting from the loss of dopaminergic neurons that originate in the substantia nigra pars compacta (SNpc) and innervate to the caudate putamen. Hallmarks of PD include neuroinflammation and accumulation of misfolded and modified self-proteins, mostly α–synuclein, as intraneuronal inclusions or Lewy bodies. Neuroinflammation is mediated by microglia which are activated by release of misfolded and modified α-synuclein to the surrounding brain tissues following neuronal death. Activated microglia secrete proinflammatory cytokines and mediators which increase inflammation, and oxidative modification and accumulation of self-proteins, and neuronal toxicity. Additionally, modified α-synuclein and proinflammatory mediators traffic to the lymph nodes and induce effector T cells (Teffs) that recognize modified epitopes on α-synuclein. We showed that CD4+ T helper type 1 and type 17 (Th1 and Th17) cells traffic back to the inflammatory foci in the brain and exacerbate the cycle of neuroinflammation-protein misfolding-neurodegeneration by hyper-activating microglia to higher states of neurotoxicity. In an animal model of PD, Th17 Teffs proved to exacerbate neurotoxicity to greater levels than Th1 cells, however the mechanism by which those Teff lineages function remain enigmatic. To determine whether these Teff types were killing neurons either through direct-mediated killing mechanisms or by indirectly hyper-activing microglia to neurotoxic levels, we developed three long-term stable Teff clones that recognize nitrated-α-synuclein (N-α-syn) as a modified self-antigen present in PD. These were phenotypically and functionally characterized as Th1, Th17, and a hybrid Th17.1 clonotypes, and displayed increasing dopaminergic neurotoxicity associated with their phenotype, respectively.

PURPOSE

The purpose of these studies was to assess differential cytokine responses by BV-2 microglia co-cultured with each activated Th1, Th17, or Th17.1 N-α-syn specific, clonotypic Teff.

METHODS

T cell lines and BV-2 microglia culture. Cells of the microglial cell line BV-2 were cultured in complete DMEM supplemented with 5% FBS. Effector T cell (Teff) lines were derived from mice immunized and boosted with nitrated-α-synuclein (N-α-syn). Lines were cloned and maintained in excess of 8 months by weekly subculture in complete RMPI-1640 supplemented with 10% FBS, 30 µg/ml of N-α-syn, 20 x 10^6 irradiated syngeneic APCs, and a cocktail of Teff-type selection cytokines and antibodies (Reynolds et al., 2010). Media for Th1 Teffs was supplemented with 25 U/ml IL-2; for Th17 Teffs media contained TGF-β, IL-6, IL-1β, IL-23, anti-IL-4, anti-IFN-γ, and anti-IL-2; and for Th17.1, media contained TGF-β, IL-6, IL-1β, IL-23, and IL-2 (Wilshusen et al., unpublished results)

Th1, Th17, and Th17.1 were activated for 30 minutes by stimulation with anti-CD3/anti-CD28 transactivation beads (Dynabeads) at a 1:1 ratio of bead:cell and excess beads removed. Activated Teffs were added to BV-2 microglia and co-cultured at a 1:1 ratio of T cell:microglia. Teffs from each T cell line, Th1, Th17, and Th17.1, were co-cultured for with BV-2 microglia in duplicate for 24 hours, after which Teffs and supernatant were removed, fresh media replaced, and adherent BV-2 microglia cultured for 24 hours before removing culture supernatants for evaluation of cytokine levels. BV-2 microglia cultured in the absence of Teffs served as controls.

Cytokine array assay. Supernatants from BV-2 microglia treated with activated Th1, Th17, Th17.1 or media alone (Control) were analyzed for cytokine type and relative level by cytokine membrane assay (Mouse Inflammatory Antibody Array, ab197473-40, AbCam, Cambridge, MA) containing cytokine targets and controls performed by the manufacturer's instructions. Membranes were visualized with fluorescence probes and relative fluorescence intensities were measured using a microarray scanner (GenePix 4000B Microarray Scanner, Molecular Devices). Relative intensities were determined by digital image analysis using Image J (National Institutes of Health)

Statistics. Significant differences in means of relative intensities of cytokine measurements were determined for control-, Th1-, Th17-, or Th17.1-treated BV-2 microglia by one way ANOVA with Fisher's LSD post hoc analyses. P values were adjusted to control for false discovery rate by the procedure of Benjamini, Krieger, and Yekutieli.

RESULTS

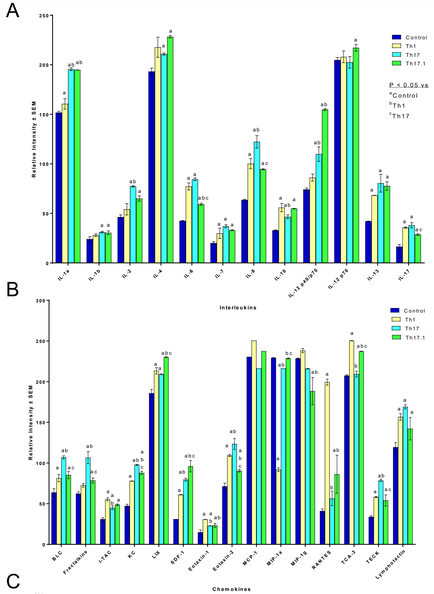

Figure 1. Cytokines from Teff-treated BV-2 microglia. BV-2 microglia were co-cultured with activated Th1, Th17, or Th17.1 Teff clones specific for N-a-synuclein, or remained untreated (Control). After Teffs and supernatants were removed, BV-2 microglia were cultured for 24 hours and supernatant assessed for expression of cytokines by antibody capture membrane array for (**A**) interleukins (**B**) chemokines, and (**C**) the factor group including TNF, IFN, and CSF families (Mouse Inflammatory Antibody Array, ab197473-40, AbCam). Significant differences between means (± SEM) for relative intensities of cytokine responses, where P < 0.05 compared to BV-2 microglia cultured with [a]media (Control), [b]Th1 Teffs, or [c]Th17 Teffs.

Compared to controls, treatment with **Th1 Teffs**, significantly increased levels of 8/12 interleukins (**A**), 12/14 chemokines (**B**), and 10/13 group factors (**C**). Treatment with **Th17 Teffs** increased levels in 11/12 interleukins (**A**), 11/14 chemokines (**B**), and 12/13 grouped factors (**C**). Treatment with **Th17.1 Teffs** increased levels in 12/12 interleukins (**A**), 13/14 chemokines (**B**), and 12/13 grouped factors (**C**)

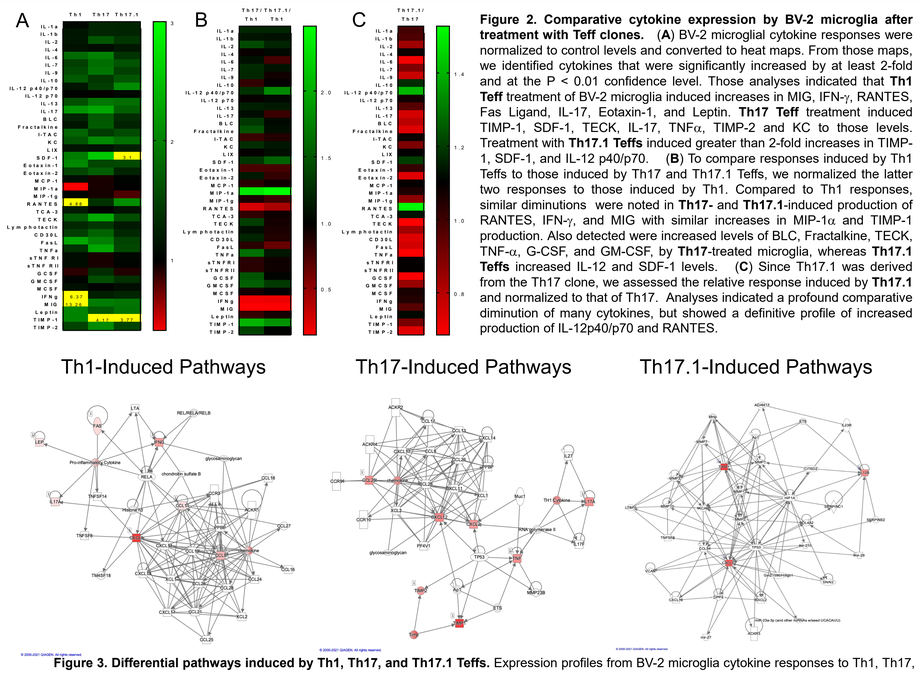

Figure 2. Comparative cytokine expression by BV-2 microglia after treatment with Teff clones. (**A**) BV-2 microglial cytokine responses were normalized to control levels and converted to heat maps. From those maps, we identified cytokines that were significantly increased by at least 2-fold and at the P < 0.01 confidence level. Those analyses indicated that **Th1 Teff** treatment of BV-2 microglia induced increases in MIG, IFN-γ, RANTES, Fas Ligand, IL-17, Eotaxin-1, and Leptin. **Th17 Teff** treatment induced TIMP-1, SDF-1, TECK, IL-17, TNFα, TIMP-2 and KC to those levels. Treatment with **Th17.1 Teffs** induced greater than 2-fold increases in TIMP-1, SDF-1, and IL-12 p40/p70. (**B**) To compare responses induced by Th1 Teffs to those induced by Th17 and Th17.1 Teffs, we normalized the latter two responses to those induced by Th1. Compared to Th1 responses, similar diminutions were noted in **Th17-** and **Th17.1**-induced production of RANTES, IFN-γ, and MIG with similar increases in MIP-1α and TIMP-1 production. Also detected were increased levels of BLC, Fractalkine, TECK, TNF-α, G-CSF, and GM-CSF, by **Th17**-treated microglia, whereas **Th17.1 Teffs** increased IL-12 and SDF-1 levels. (**C**) Since Th17.1 was derived from the Th17 clone, we assessed the relative response induced by **Th17.1** and normalized to that of Th17. Analyses indicated a profound comparative diminution of many cytokines, but showed a definitive profile of increased production of IL-12p40/p70 and RANTES.

Figure 3. Differential pathways induced by Th1, Th17, and Th17.1 Teffs. Expression profiles from BV-2 microglia cytokine responses to Th1, Th17, and Th17.1 Teffs were normalized to Control (**Figure 2A**). Profiles of cytokines that were expressed above or below 2-fold and at significance levels below 0.01 were entered into and evaluated by Ingenuity Pathway Analysis (IPA). Overall, Th1 and Th17 upregulated similar network pathways including cellular movement, hematological system development and function, and immune cell trafficking. Thus, increasing the stimulation and production of further inflammatory mediating cells and increasing chemotaxis and tissue extravasation of these inflammatory cells. Interestingly, Th17.1 upregulated pathways associated with connective tissue disorders, inflammatory diseases, and organismal injury and abnormalities.

Th1-Induced Pathways. For Th1 treated BV-2 microglia, MIG (CXCL9) displayed the most substantial expression change at a 13.3 relative to control, with IFN-γ, RANTES (CCL5), Fas Ligand, IL-17, Eotaxin (CCL11) and Leptin completing the profile of response that is programed to promote MIHC presentation, chemotaxis of other leukocytes, killing activity, induction of apoptosis.

Th17- and Th17.1-Induced Pathways. Pathways for Th17- and Th17.1-treated BV-2 microglia showed high levels of TIMP-1 and SDF-1. TIMP-1 is a matrix metalloproteinase inhibitor that is associated with attenuation of inflammatory responses and may play a compensatory regulatory role. Stromal cell-derived factor 1 (SDF-1, CXCL12) is a potent chemotactic agent for lymphocytes and has been shown to play a role in neurogenesis and neuroinflammation.

Co-culture with Th17 Teffs induced BV-2 microglia to produce TECK (CCL25) and KC (CXCL1); both chemotactic factors for macrophages/dendritic cells and neutrophils, respectively. **Th17 Teffs** also induced TNFα a known proinflammatory cytokine and inducer of apoptosis. Microglial-derived IL-17 induces other chemokines and exacerbates neuroinflammation and with TNFα kills dopaminergic neurons.

Co-culture with Th17.1 Teffs induced BV-2 microglial production to not only secrete TIMP-1 and SDF1 as with Th17 Teffs, but in contrast induced also production of RANTES and IL12p40/p70 as was induced by **Th1 Teffs**.

CONCLUSIONS

- This study confirms that Th1 and Th17 Teffs that recognize the modified self protein N-α-synuclein upregulates proinflammatory cytokine production by BV-2 microglia and demonstrates the proinflammatory potential for a novel Th17.1 Teff clone.
- Co-culture of clonotypic Teffs produced differential cytokines responses by BV-2 microglia. Th1 Teffs led to higher expression of IFN-γ, RANTES, and MIG.
- Th17 or Th17.1 co-culture led to higher expression of TIMP-1 and SDF-1, while Th17 yielded higher expression of TECK, IL-17, TNF- α, and KC (CXCL1), whereas Th17.1 induced higher levels of RANTES and IL-12 p40/p70 simiar to Th1 Teffs. .
- IPA demonstrated microglial pathways increased in cellular movement, hematological system development and function, and immune cell trafficking for Th1 and Th17 Teffs, while Th17.1 treatment yielded pathways related to disorder of connective tissue, inflammation, and organismal injury.
- Future studies from these observations may identify and target specific cytokine pathways targeted to inhibit neuroinflammation and neuronal cell death.