Human T_H17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation

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Abstract

T_H17 lymphocytes appear to be essential in the pathogenesis of numerous inflammatory diseases. We demonstrate here the expression of IL-17 and IL-22 receptors on blood-brain barrier endothelial cells (BBB-ECs) in multiple sclerosis lesions, and show that IL-17 and IL-22 disrupt BBB tight junctions *in vitro* and *in vivo*. Furthermore, T_H17 lymphocytes transmigrate efficiently across BBB-ECs, highly express granzyme B, kill human neurons and promote central nervous system inflammation through CD4⁺ lymphocyte recruitment.

T-helper type 1 (T_H1)^{1,2} and type 17 (T_H17) lymphocytes contribute to autoimmune inflammatory diseases³ including multiple sclerosis and its mouse model, experimental autoimmune encephalomyelitis (EAE)^{4,5}. Disruption of the BBB and trafficking of autoreactive T cells from the systemic compartment into the central nervous system (CNS) are important, early events in the development of multiple sclerosis lesions⁶. In support of T_H1 lymphocytes have been shown to migrate efficiently across the human BBB^{7,8}. To evaluate T_H17 lymphocyte migration to the brain relative to T_H1 cells, we employed an *in vitro* model of the human BBB using human brain-derived microvascular endothelial cells. We generated human T_H1 and T_H17 lymphocytes *in vitro* using peripheral blood CD4⁺ lymphocytes cultured with IL-12 and IL-23, respectively (Supplementary Methods online). Human T_H17 lymphocytes migrated more avidly across the BBB than did T_H1 or freshly isolated (*ex vivo*) CD4⁺ lymphocytes (Fig. 1a, *P* < 0.01). To ensure that the selective

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Note: Supplementary information is available on the Nature Medicine website.

AUTHOR CONTRIBUTIONS

H.K. conducted most of the experiments; K.K. performed and analyzed animal studies; I.I. and A.D.-D. contributed to immunostaining and *in vitro* protocols; R.C. assisted with confocal microscopy and performed some EAE experiments; M.B. assisted with BBB-EC isolation and culture; F.G. performed the killing assay; N.A. provided critical input on data analysis; B.B. designed and supervised the animal studies; H.K. and A.P. designed the study, analyzed the data and wrote the manuscript; A.P. secured the funding.

accumulation of T_H17 lymphocytes indeed reflects the preferential transmigration ability of T_H17 cells, we analyzed the intracellular cytokine profile of the cell population before and after migration across BBB-ECs, looking specifically at IL-17 and at IL-22, a recently identified cytokine product of T_H17 cells^{9–11}. We noted a significant enrichment in the number of IL-17⁺ and IL-22⁺ expressing CD4⁺CD45RO⁺ memory lymphocytes upon migration across the BBB (Fig. 1b, $P < 0.001$ for IL-17⁺ and $P < 0.05$ for IL-22⁺ cells, $n = 3$), confirming the ability of T_H17 lymphocytes to cross the BBB *in vitro*. To further substantiate these observations, we generated myelin oligodendrocyte glycoprotein (MOG)-specific T_H1 and T_H17 lymphocytes from 2D2 mice *in vitro* and transferred these separately into T and B lymphocyte-deficient *Rag1*^{−/−} mice. Equal numbers of T_H cells were found in the CNS of *Rag1*^{−/−} mice 7 d after transfer, regardless of whether donor cells were polarized into T_H1 or T_H17 cells, confirming that T_H1 and T_H17 cells primed and expanded in the periphery access the CNS *in vivo* (Fig. 1c). To validate these human *in vitro* and mouse *in vivo* observations, brain sections from humans with multiple sclerosis and from unaffected controls were immunostained for CD45RO and IL-17 or IL-22. Numerous CD45RO⁺ cells immunopositive for IL-17 or IL-22 were detected in highly infiltrated multiple sclerosis lesions, but not in normal-appearing white matter or non-inflamed brain specimens (Fig. 1d,e). Taken together, these results emphasize the potential importance of T_H17 lymphocyte infiltration into the CNS and these lymphocytes' consequent involvement in lesion formation in multiple sclerosis and EAE.

So far, the encephalitogenic activity of T_H17 cells has been attributed to IL-17 (refs. 12,13). To investigate whether the action of T_H17 cells extends beyond the proinflammatory influence of IL-17, we explored the possibility that T_H17 cells might express cytolytic molecules and therefore analyzed T_H17 cells for the expression of perforin, granzyme A and B. Notably, whereas virtually no *ex vivo* CD4⁺CD45RO⁺ cells produced cytolytic enzymes (data not shown), granzyme B was expressed in as many as 22.5% of IL-17-producing CD4⁺CD45RO⁺ cells and 17.3% of IL-22⁺ lymphocytes after 6 d of culture with IL-23 (Fig. 1f). Even more striking is that 60% of cells coexpressing IL-17 and IL-22 also expressed granzyme B (Fig. 1g). We therefore tested the capacity of granzyme B⁺ T_H17 cells to kill human fetal neuron-enriched cultures and found that they showed considerable cytolytic activity ($32.83 \pm 2.54\%$) as compared to unactivated T lymphocytes ($6.15 \pm 2.37\%$) (Fig. 1h, $P < 0.0001$, $n = 3$).

We next analyzed IL-17 receptor (IL-17R) and IL-22 receptor (IL-22R) expression on human BBB-ECs, and investigated whether IL-17 and IL-22 influence BBB integrity. IL-17R and IL-22R were detected on the surface of a subset of human BBB-ECs in primary culture (Fig. 2a; 23% of IL-17R⁺ and 16% of IL-22R⁺). *In situ*, however, IL-17R and IL-22R were undetectable in CNS material from subjects without multiple sclerosis. However, both receptors were strongly expressed on CNS vessels within heavily infiltrated multiple sclerosis lesions, colocalized with caveolin-1, a marker of brain endothelial cells (Fig. 2b,c).

We further investigated whether brain endothelial IL-17R and IL-22R were functional, and whether IL-17 and IL-22 could affect BBB permeability. Addition of 10 ng/ml of IL-17 or IL-22 to monolayers of human BBB-ECs induced a marked and sustained increase in the

diffusion of fluorescence-labeled BSA (Fig. 2d). This effect was dose dependent, reached a plateau at 100 ng/ml and coincided, for IL-17, with a decrease in the expression of occludin and zonula occludens (ZO)-1, two important tight junction-associated molecules (Fig. 2e). A similar reduction of occludin, and to a lesser extent ZO-1, expression was demonstrated by western blotting in spinal cord homogenates from EAE mice (Fig. 2f). *In situ* staining further confirmed a decrease in ZO-1 immunoreactivity in cerebellar lesions of MOG-immunized mice (Fig. 2f). These results are in line with our recent data showing a disruption of tight-junction proteins in highly infiltrated vessels of multiple sclerosis lesions¹⁴. The exact mechanism mediating IL-22-induced BBB permeability remains uncertain, however.

We next explored the capacity of IL-17 and IL-22 to modulate lymphocyte migration across human BBB-ECs and found that IL-17 and IL-22 promote transmigration of human *ex vivo* CD4⁺ lymphocytes (Fig. 2g), most likely through enhanced BBB-EC-mediated secretion of CCL2 (or MCP-1) (Fig. 2h). IL-17 also induced IL-6 and CXCL8 (or IL-8) expression by BBB-ECs, whereas expression of TGF- β , ICAM-1, VCAM-1, CCL5 (or RANTES) and CXCL10 (or IP-10) remained unaffected (data not shown). Taken together, these results strongly suggest that T_H17 cells, through the action of IL-17 and IL-22, play a unique role in permeabilizing the human BBB both to soluble molecules and to circulating CD4⁺ lymphocytes.

Our study further refines the phenotype of human T_H17 lymphocytes as cells coexpressing IL-17, IL-22 and granzyme B, and provides strong evidence that IL-17 and IL-22 induce a breach in the BBB and promote the recruitment of additional CD4⁺ lymphocytes. Although IL-22 has the potential to influence the ability of T_H17 lymphocytes to gain access to the CNS, *in vivo* evidence indicates that IL-22 does not directly affect their encephalitogenicity (B.B., personal communication). We postulate that T_H17 cells produce multiple mediators contributing to their highly encephalitogenic potential, among them cytolytic enzymes such as granzyme B.

Supplementary Material

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Figure 1.

T_H17 lymphocytes migrate efficiently across the BBB *in vitro* and *in vivo* and kill human neurons. **(a)** Human CD4⁺CD45RO⁺ T_H17, CD4⁺ T_H1 (both generated *in vitro*, see Supplementary Methods) and *ex vivo* CD4⁺ lymphocytes were allowed to migrate across human BBB-ECs in a modified Boyden chamber assay (ref. 14 and Supplementary Methods) for 18 h. Significantly more T_H17 lymphocytes migrated than either T_H1 or *ex vivo* CD4⁺ lymphocytes. **(b)** CD4⁺CD45RO⁺ T_H17 lymphocytes were allowed to migrate across human BBB-ECs for 18 h. Cells were stained for IL-17 and IL-22 before and after migration. The cytokine profile revealed the preferential migration of IL-17⁺ and IL-22⁺ lymphocytes. **(c)** Immune cells from lymph nodes and spleen of MOG_{35–55}-immunized 2D2 mice were polarized toward T_H1 or T_H17 and transferred to *Rag1*^{-/-} mice, and CD45^{hi}CD4⁺ lymphocytes were isolated from the CNS 7 d after transfer. Shown is a representative flow cytometry dot plot of CNS cell content from *Rag1*^{-/-} mice injected with either T_H1- (left) or T_H17-polarized (right) 2D2 lymphocytes ($n = 4$ mice per group). **(d)** Human CNS postmortem material from unaffected individuals (control, non-inflamed; above) and heavily infiltrated CNS material from individuals with multiple sclerosis (below) were immunostained for CD45RO (red), IL-17 (green) and nuclear stain TO-PRO3 (blue). Confocal microscopy imaging confirmed the presence of IL-17⁺CD45RO⁺ cells (yellow) in infiltrated multiple sclerosis lesions but not in control CNS. Bar, 75 μ m. **(e)** Similarly,

IL-22⁺CD45RO⁺ staining was observed in multiple sclerosis lesions, but not in control CNS material. **(f)** Human CD4⁺CD45RO⁺ T_H17-polarized lymphocytes were stained for CD45RO, IL-17, IL-22 and granzyme B. Both IL-17⁺ and IL-22⁺-producing lymphocytes expressed granzyme B (22.5% and 17.3%, respectively). **(g)** More than 60% of IL-17⁺IL-22⁺ lymphocytes highly expressed granzyme B. Granzyme A and perforin were not detected in T_H17 cells, whether or not these cells produced IL-22 (data not shown). **(h)** The cytotoxic activity of T_H17 lymphocytes was assessed using neuron-enriched cultures obtained from human fetal CNS material and compared to that of unactivated T lymphocytes. All data shown are representative of the mean $\pm$ s.e.m. of three independent experiments.

Figure 2.

IL-17 and IL-22 receptors are expressed on human brain endothelium, and their activation permeabilizes the BBB. **(a)** Unactivated human BBB-ECs grown in primary culture were stained for IL-17R and IL-22R, revealing their expression on the surface of 23.2% and 16.6% of BBB-ECs, respectively. **(b)** Human CNS postmortem material from unaffected individuals (control, non-inflamed; above) and heavily infiltrated CNS material from individuals with multiple sclerosis (below) were immunostained for IL-17R (green), caveolin-1 (red) and nuclear stain TO-PRO3 (blue). Confocal microscopy imaging confirmed the expression of IL-17R on caveolin-1⁺ endothelium in inflamed CNS material. IL-17R expression was undetectable in control CNS material. Bar, 75 μ m. **(c)** Similarly, IL-22R staining was observed on endothelial cells in multiple sclerosis lesions, but not in controls. **(d)** Human BBB-ECs were grown in Boyden chambers and treated with IL-17 (top) or IL-22 (bottom). Permeability of the monolayers was monitored with fluorescent BSA, showing that BBB-EC monolayer permeability increased after treatment with either IL-17 or IL-22. **(e)** Western blot for the tight-junction proteins occludin, ZO-1 and junction adhesion molecule (JAM)-1 from human BBB-ECs revealed disruption of occludin and ZO-1 by IL-17 (100 ng/ml, 18 h). **(f)** Western blot for tight-junction proteins in spinal cord homogenates of MOG₃₅₋₅₅-immunized EAE mice revealed a similar reduction in occludin and ZO-1. *In situ* immunostaining for ZO-1 (red) and nuclear stain TO-PRO3 (blue) in normal-appearing cerebellar white matter (control) and in infiltrated and demyelinated cerebellar lesions from C57BL/6 mice immunized with MOG₃₅₋₅₅ (EAE, grade 4). Confocal microscopy imaging confirmed disruption of ZO-1 around infiltrated vessels. Bar, 75 μ m. **(g)** Freshly isolated peripheral blood human CD4⁺ lymphocytes were allowed to migrate for 18 h across IL-17- (100 ng/ml) or IL-22-treated (100 ng/ml) human BBB-ECs. Both cytokines

promoted migration of human *ex vivo* CD4⁺ lymphocytes across human BBB-ECs, as compared to control. **(h)** CCL2 (or MCP-1) secretion by human BBB-ECs was assessed by ELISA in untreated and IL-17– or IL-22–treated cultures (100 ng/ml, 18 h). Both IL-17 and IL-22 upregulate CCL2 secretion by human BBB-ECs. All data shown represent the mean $\pm$ s.e.m. from three independent experiments performed in triplicate.