Arch. Biol. Sci., Belgrade, 61 (1), 29-36, 2009 DOI:10.2298/ABS0901029M

MURINE BRAIN ENDOTHELIAL CELLS DIFFERENTLY MODULATE INTERFERON-γ AND INTERLEUKIN-17 PRODUCTION *IN VITRO*

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Abstract — Brain endothelial cells (BEC) are the major constituents of the blood-brain barrier (BBB), the structure that controls entrance of immune cells into CNS parenchyma. Our aim was to investigate the influence of BEC on production of IL-17 and IFN-γ–cytokines that are important for CNS inflammation. To that end, co-cultivations of the bEnd.3 brain endothelial cell line and lymph node cells (LNC) were performed, and gene expression and production of IL-17 and IFN-γ were determined. It was found that bEnd.3 cells inhibited expression and production of IFN-γ, but not of IL-17. Additionally, bEnd.3 cells also reduced production of the major IFN-γ-promoting cytokine – IL-12 – in LNC. The observed variation in modulation of pro-inflammatory cytokines by BEC could be of importance for the understanding of CNS inflammation.

Key words: CNS, cytokine, inflammation, bEnd.3, lymph node, concanavalin A

UDC 577.25:612.017:577.27

INTRODUCTION

Brain endothelial cells (BEC), highly specialized endothelial cells, are the main constituents of the blood-brain barrier (BBB). Structural specializations of BEC include highly organized tight junctions, the absence of expression of MHC II molecules, and highly developed transport systems (Prat et al., 1999). Due to these characteristics, BEC have a decisive role in the selection of substances and cells transported in or out of the CNS. In connection with migration of cells to the CNS, BEC display various adhesive molecules on their surface, such as VCAM-1, ICAM-1, PECAM-1, ICAM-2, CCL19, and E and P selectin in physiological and pathological conditions (Engelhardt and Ransohoff, 2005; Ranshoff et al., 2003, Gavins et al., 2007), which allow them to communicate directly with leukocytes involved in normal immune surveillance or with activated cells infiltrating the CNS. Activated T cells can cross the compact BBB, and if they recognize target structure(s) in the CNS, as a consequence various soluble factors are released that cause BEC to become less restrictive, leading to increased lymphocyte trafficking across the BBB as an early event in inflammatory CNS diseases (Archambault et al., 2005; Becher et al., 2006). Besides controlling transport of leukocytes across the BBB, BEC are also able to influence immune functions of CNS resident cells and blood-borne cells through secretion of different substances, including nitric oxide (NO), prostaglandins, and cytokines such as IL-6, IL-8, MCP, RANTES, TNF-α, IL-1, GM-CSF, IL-10, and endothelin-1 (Zhao et al., 2006; Konsman et al., 2007; Matsumura and Kobayashi, 2004; Reyes et al., 1999).

The pro-inflammatory T cell cytokines IFN-γ and IL-17 are considered to be among major culprits in the CNS inflammatory disease multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE) (Segal, 2005; Wheeler and Owens, 2005). A pivotal Th1 cytokine, IFN-γ activates macrophages and up-regulates production of a variety of pro-inflammatory mediators such as IL-12, IL-15, TNF-α, interferon-inducible protein-10, and inducible NO synthase (iNOS) in different cell types. It is known that IFN-γ–secreting

Th1 cells are present in the CNS before the onset of clinical symptoms and disappear during the remission phase of EAE (Juedes et al., 2000; Renno et al., 1995). They are also present in MS lesions (Traugott et al., 1983). Treatment with IFN-γ exacerbates MS (Panitch et al., 1987), and mice deficient in T-bet – master regulator of Th1 differentiation – are resistant to EAE (Nath et al., 2006). Moreover, it has recently been shown that IFN-γ largely contributes to myelin destruction in a mouse model of demyelination (Mana et al., 2006). Recent data indicate that the newly discovered Th17 population is the pathogenic population in EAE pathogenesis (Aggarwal et al., 2003; Langrish et al., 2005). It has been demonstrated that Th17 cells are characterized by the production of a distinct profile of effector cytokines, including IL-17 (IL-17A), IL-17F, TNF-α, and IL-6 (Langrish et al., 2005; Betteli et al., 2008). Moreover, IL-17 is important in the induction and maintenance of inflammation, as it can induce the production of other cytokines and chemokines from a variety of cell types and coordinate the recruitment of neutrophils and monocytes to the site of inflammation (Kolls and Linden, 2004). It was shown that in MS patients, IL-17 mRNA and protein were increased in both brain lesions and mononuclear cells isolated from blood and cerebrospinal fluids (Lock et al., 2002; Matusevicius et al., 1999). Further, induction of EAE is blocked in mice deficient in IL-23, which promotes the differentiation of IL-17–producing cells (Cua et al., 2003); adoptive transfer of autoantigen specific Th17 cells induced EAE (Langrish et al., 2005); and finally, the development of EAE was significantly suppressed in IL-17 knockout mice or mice treated with anti–IL-17 vaccine (Komiyama et al., 2006; Wraith, 2006).

In light of the intimate relationship between BEC and peripherally activated T lymphocytes and the importance of IFN-γ and IL-17 for the pathogenesis of MS and EAE, our aim was to investigate if BEC can influence production of these cytokines in T lymphocytes. We report here that BEC potently modulate IFN-γ (but not IL-17) gene expression and secretion. This influence could be of potential importance for regulation of the auto-aggressive T cell response.

MATERIALS AND METHODS

Murine brain endothelial cell line bEnd.3 was obtained from the American Type Culture Collection (ATTC, MD, USA). The cells were grown in HEPES-buffered RPMI-1640 medium (Sigma, Germany) containing 10% heat-inactivated fetal bovine serum (PAA Chemicals, Austria), antibiotic/antimycotic, and 2 mM glutamine. Cells from 22 to 25 passages were used for all experiments. Cervical lymph nodes (LN) were isolated from BALB/c mice and mashed through steel net into the medium. The resulting cell suspension (LNC) was centrifuged and resuspended for final counting and use in experiments. Adequate measures were taken to minimize pain or discomfort of the animals. For the experiments with co-cultivation, bEnd.3 cells ($2x10^5$/well) were seeded in 24-well plates (Sarsted, Germany) and incubated overnight. Subsequently, LNC ($4x10^6$/well) were added to cultures, either directly onto bEnd.3 or into tissue culture inserts (0.2 μM Anapore membrane, Nunc, Denmark) that prevented contact between these two cell populations. The cells were cultivated for 6 or 24 h under standard conditions (5% CO_2, 37°C) in the presence or absence of 2.5 μg/ml of concanavalin A (ConA, Pharmacia, Sweden). Afterwards, cell culture supernatants were collected and frozen until used in ELISA (mouse IL-17, IFN-γ, and IL-12 ELISA DuoSets, R&D Systems, MN, USA), and the pelleted cells were used for total RNA isolation and subsequent real time (RT)-PCR. Ribonucleic acid was reverse transcribed using random hexamer primers and MMLV reverse transcriptase (Fermentas, Lithuania). The RT-PCR procedure was performed in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA) using the TaqMan Universal PCR Master Mix (Applied Biosystems). Thermocycler conditions comprised the initial step (50°C for 2 min and subsequent heating to 95°C for 10 min). This was followed by a two-step PCR program at 95°C for 15 sec and 60°C for 60 sec for 40 cycles. The PCR primers and probes used were as follows: IFN-γ forward primer 5'-TGG CAT AGA TGT GGA AGA AAA GAG -3'; IFN-γ - reverse primer 5'-TGC AGG ATT TTC ATG TCA CCA T-3'; IFN-γ probe FAM 5'-TTT TGC CAG TTC CTC CAG ATA TCC AAG

AAG A-3' TAMRA; IL-17 forward primer 5'-ATC AGG ACG CGC AAA CAT G-3'; IL-17 reverse primer 5'-TGA TCG CTG CTG CCT TCA C -3'; IL-17 probe FAM 5'-CTT CAT CTG TGT CTC TGA TGC TGT TGC TGC-3' TAMRA; β-actin forward primer 5'-GCT TCT TTG CAG CTC CTT CGT-3'; β-actin reverse primer 5'-CCA GCG CAG CGA TAT CG-3'; and β-actin probe VIC 5'-CAC CCG CCA CCA GTT CGC CAT-3' TAMRA. Expression of IL-17 and that of IFN-γ were determined relative to expression of β-actin. Relative gene expression in cultures grown in a medium without additional treatment was adopted as the value of an arbitrary unit. The results are presented as means ± SD of values obtained in repeated experiments. Student's t test was performed for statistical analysis of the differences observed. A p value less than 0.05 was considered significant.

Abbreviations: CNS – central nervous system; ConA – concanavalin A; EAE – experimental autoimmune encephalomyelitis; IFN – interferon; IL – interleukin; LNC – lymph node cells; MHC – major histocompatibility complex; MS – multiple sclerosis; Th – helper T cells.

RESULTS

Inasmuch as interaction of BEC and T cells migrating to the CNS parenchyma is important for the initiation of inflammation in the CNS, we examined the influence of bEnd.3 cells on IFN-γ and IL-17 generation in T cells. Production of IFN-γ in LNC was markedly inhibited in the presence of bEnd.3 cells after 6 or 24 h of cultivation (Fig. 1). A stronger effect was observed if the contact between cells was inhibited by a tissue culture insert. On the other hand, IL-17 release from LNC was just weakly modified in the presence of bEnd.3 (Fig. 1). The observed effect on cytokine production was not a consequence of decline in the number of LNC in co-cultivation, as was deduced from cell viability and cell number determination by MTT assay and trypan blue staining, respectively (data not shown). In order to explore if the observed influence of bEnd.3 on cytokine secretion by LNC was realized through gene expression modulation, LNC from co-cultivations in the presence of tissue culture inserts were centrifuged, RNA from pelleted cells was isolated, and RT-PCR analysis was performed. To judge from cytokine secretion, mRNA expression of IFN-γ (but not IL-17) was markedly down-regulated in the presence of bEnd.3 cells after 6 or 24 h of cultivation (Fig. 2). Thus, the influence of b.End3 cells on production of IFN-γ was most likely mediated through inhibition of its gene expression. Importantly, there was a marked difference in the influence of bEnd.3 on IFN-γ and IL-17, as these cells strongly inhibited the former but had only a minor (if any) effect on the latter.

Having in mind that IL-12 is a well-known promoter of IFN-γ synthesis, we explored the influence of bEnd.3 cells on IL-12 production in order to find out if the observed inhibitory effect of bEnd.3 cells on IFN-γ production and gene expression was achieved through inhibition of IL-12 production. Significant inhibition of IL-12 production in co-cultivations was observed when the cells were separated with a tissue culture insert (Fig. 3). Interestingly, in the presence of direct cell-cell contact between these cells, there was no inhibition of IL-12 secretion. At the same time, bEnd.3 cells did not produce detectable amounts of IL-12 in our experiments. Almost the exact same results were obtained in co-cultures lasting for 6 and 24 h, thus clearly suggesting that there was a correlation between IFN-γ and IL-12 secretion in the absence (but not in the presence) of direct contact between bEnd.3 cells and LNC.

DISCUSSION

This study attempted to clarify if BEC can affect production of marker Th1 and Th17 cytokines, IFN-γ and IL-17, respectively. The bEnd.3 cells used in the investigation strongly reduced IFN-γ (but not IL-17) production *in vitro*, thus implying a cytokine-specific effect of brain endothelial cells on lymphocytes.

Although there are numerous data describing the influence of T cells and their cytokines on brain endothelium, and the function of BBB in particular (Engelhardt and Ransohoff, 2005), investigations of the opposite process are not so abundant. Regarding IFN-γ, there is a paper that seems to contradict our work, as it reports that human BEC do not influ-

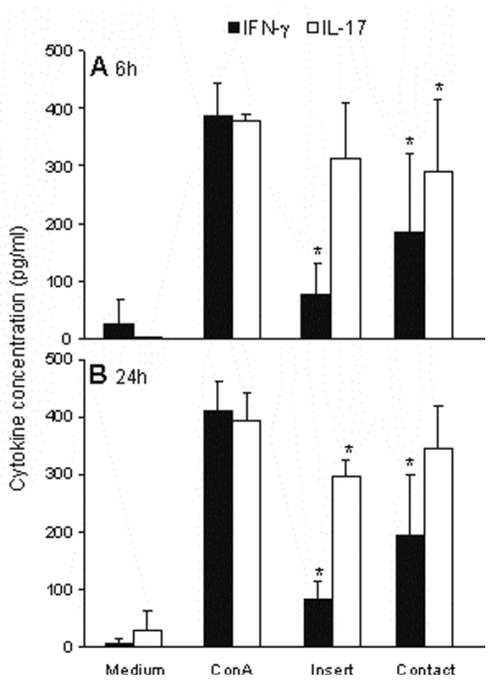

Fig. 1. Influence of bEnd.3 cells on IFN-γ and IL-17 production in ConA-stimulated LNC. Lymph node cells were cultivated alone or co-cultivated with bEnd.3 cells for 6 h (A) or 24 h (B) in the absence (medium) or presence of ConA (2.5 μg/ml) with (insert) or without (contact) a tissue culture insert. Cell-free culture supernatants were subsequently collected and analyzed for the concentration of IL-17 and IFN-γ by ELISA. Data obtained from 3-7 experiments are presented as means ± SD of values obtained in individual experiments. *p<0.05 represents statistical significance of the difference in relation to cultures of LNC cultivated alone and stimulated with ConA.

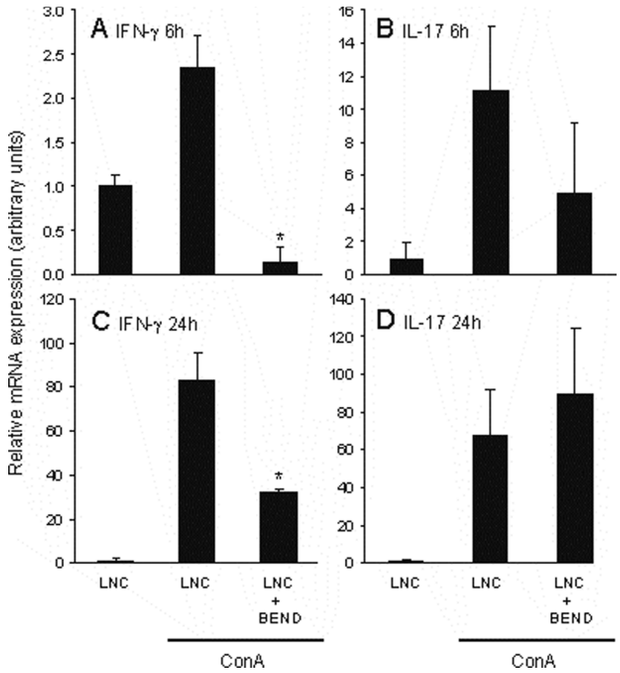

Fig. 2. Influence of bEnd.3 cells on IFN-γ and IL-17 gene expression in ConA-stimulated LNC. Lymph node cells (2x105/well) were cultivated with or without ConA (2.5 μg/ml) in the absence or presence of bEND.3. In co-cultivations, bEND and LNC were separated by tissue-culture inserts. After 6 h (A, B) and 24 h (C, D) of cultivation, RNA was isolated, and reverse transcription followed by RT- PCR analysis for IFN-γ (A, C) and IL-17 (B, D) was performed. Data obtained from two experiments are presented as means ± SD of values obtained in individual experiments. *p<0.05 represents statistical significance of the difference in relation to cultures of LNC cultivated alone and stimulated with ConA.

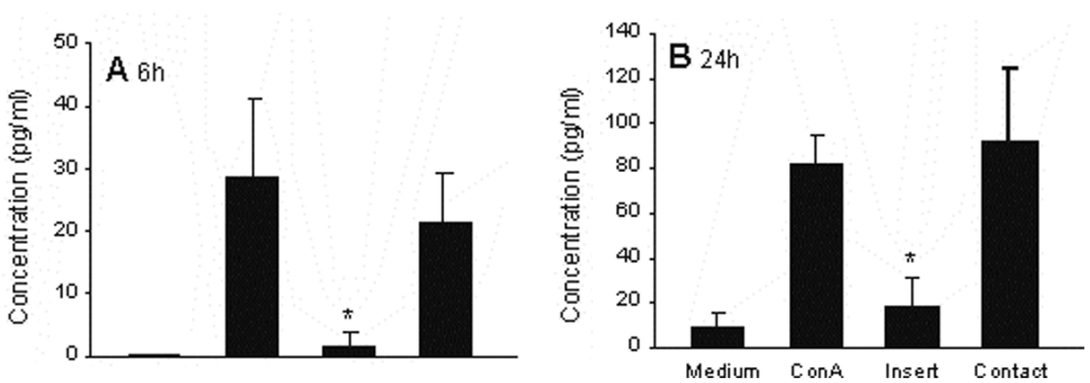

Fig. 3. Influence of bEnd.3 cells on IL-12 production in ConA-stimulated LNC. Lymph node cells were cultivated alone or co-cultivated with bEnd.3 cells for 6 h (A) or 24 h (B) in the absence (medium) or presence of ConA (2.5 μg/ml) with (insert) or without (contact) a tissue culture insert. Cell-free culture supernatants were subsequently collected and analyzed for the concentration of IL-12. Data obtained from 3-5 experiments are presented as means ± SD of values obtained in individual experiments. *p<0.05 represents statistical significance of the difference in relation to cultures of LNC cultivated alone and stimulated with ConA.

ence IFN-γ production in Th1 cells (Biernacki et al., 2001). However, differences in the species, purity of lymphocytes used, and stimulation employed in the two studies could easily account for the discrepancy in data. In their study, Biernacki et al. (2001) purified Th1 cells from peripheral blood of healthy subjects and stimulated them with myelin basic protein (MBP). The obvious advantages of their system are purity of the used populations and antigen specificity of the T cell response. On the other hand, using a mixed population isolated from lymph nodes and stimulation with a polyclonal T cell activator (ConA) more clearly elucidates the composition of cells infiltrating CNS and the inflammatory milieu in later phases of the autoimmune process in the CNS. Whatever the case, here we record a pronounced effect of BEC on IFN-γ production. Interestingly, the reduction in IFN-γ correlated with reduction of IL-12 only in the event that bEnd.3 cells and LNC were separated by tissue culture inserts. It is possible that regulation of IL-12 production is exerted both by cell-cell contact and by soluble products, the former being largely positive and capable of attenuating the inhibitory effects of soluble factors. In the absence of these stimulatory cellular interactions, IL-12–producing cells remain exposed only to soluble factors and the net effect is inhibition of IL-12 production. It therefore seems that down-regulation of IL-12 production in co-cultivations could be responsible for the effect on IFN-γ, but only if bEnd.3 and LNC are not in direct cell-cell contact. In direct contact with LNC, bEnd.3 do not inhibit IL-12 production and have a weaker effect on IFN-γ generation. There are many other cytokines that are important for IFN-γ regulation, and bEnd.3 could affect their production as well. Inasmuch as IL-12 can be just one of the essential factors for IFN-γ synthesis, if IL-12 is inhibited there is inhibition of IFN-γ as well, but if IL-12 is not inhibited there is still room for IL-12–independent regulation of IFN-γ production to take place. This difference in the effect regarding contact between bEnd.3 and LNC could be of importance for understanding data indicating that Th1 cells (as major producers of IFN-γ) have the ability to migrate across the BBB (Biernacki et al., 2001; Prat et al., 2002), but that their encephalitogenic potential is weak in comparison to Th17 cells. Since transmigration is a complex process involving at least two crucial steps: 1. entrance into perivascular spaces, and 2. passage to CNS parenchyma (Bechmann et al., 2007), it could be that BEC in direct contact during the first step weakly influence IFN-γ production of transmigrating Th1 cells and allow their entrance into perivascular spaces, although already reducing their functionality. In the second step, BEC acting through soluble factors might reduce IFN-γ production and Th1 functionality (including encephalitogenicity) more aggressively. If we suppose that production of IFN-γ is a clear marker of Th1 functionality, the suggested speculation is even more attractive.

On the other hand, bEnd.3 had a limited (if any) effect on IL-17 generation in LNC. It therefore seems reasonable to assume that Th17 cells pass the BBB with no major effect on their functionality. Thus, this result is in accordance with the presumed importance of Th17 cells for CNS autoimmunity. There are plentiful data indicating a high encephalitogenic potential of Th17. A pivotal pathogenic role for IL-17 in the CNS autoimmune response is substantiated by attenuation of EAE with IL-17 neutralization by anti–IL-17 antibodies or in mice genetically deficient in IL-17 or the IL-17 receptor (IL-17R) (Weaver et al., 2007; Betteli et al., 2008; Kramer and Gafen, 2007). First indications about the importance of Th17 cells for the pathogenesis of MS were significant increase in IL-17 gene expression and elevation in the number of IL-17–producing CD4+ and CD8+ T cells in active lesions in comparison with silent lesions or normal tissue (Lock et al., 2002; Tzartzos et al., 2008). Importantly, it has recently been reported that human blood-brain barrier (BBB) endothelial cells in MS lesions express receptors for IL-17, and that IL-17 disrupts BBB tight junctions both *in vitro* and *in vivo* (Kebir et al., 2007). It has also been shown that Th17 lymphocytes transmigrate efficiently across BBB endothelial cells, highly express granzyme B, kill human neurons, and promote CNS inflammation through $CD4^+$ lymphocyte recruitment (Kebir et al., 2007). Taken together, these recent data clearly suggest the importance of Th17 for the pathogenesis of MS. Significantly, there is a recent finding that functional

perivascular myeloid CNS dendritic cells arise as a consequence of migration of monocytes across the human BBB through the concerted actions of BBB-secreted transforming growth factor-beta and granulocyte-macrophage colony-stimulating factor (Ifergan et al., 2008). Thus, there is the possibility that BEC even promote Th17 propagation within the CNS.

Our results clearly suggest the ability of BEC to inhibit IFN-γ and inability to interfere efficiently with IL-17 production in activated T cells *in vitro*. Such a finding is important for understanding the different encephalitogen potentials of Th1 and Th17 cells. Furthermore, it contributes to the growing body of data that should allow for the creation of more efficient therapy of neuro-inflammatory disorders.

Acknowledgments — This work was supported by the Serbian Ministry of Science (grants 143029B and 145066B). One of us (Dj. M.) received assistance in the form of a return fellowship from the Alexander von Humboldt Foundation (Bonn, Germany).

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ЋЕЛИЈЕ МИШЈЕГ МОЖДАНОГ ЕНДОТЕЛА ОСТВАРУЈУ РАЗЛИЧИТ УТИЦАЈ НА ПРОДУКЦИЈУ ИНТЕРФЕРОНА-γ И ИНТЕРЛЕУКИНА-17 *IN VITRO*

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Ћелије мождданог ендотела су основни елементи грађе крвно-мождане баријере, структуре која контролише улазак ћелија имунског система у паренхим CNS. Наш циљ је био да се испита утицај ових ћелија на продукцију интерферона-гама и интерлеукина-17, као кључних проинфламаторних цитокина у неуроинфламацији. Због тога смо вршили ко-култивацију bEnd.3 линије мождданог ендотела и ћелија лимфног чвора, и то у присуству или одсуству директног контакта међу наведеним популацијама, и одређивали експресију гена и продукцију наведених цитокина. Показало се да bEnd.3 ћелије потентно инхибирају продукцију интерферона-гама, али не и интерлеукина-17. Такође, мерили смо и продук-

цију интерлеукина-12, главног стимулаторног цитокина за продукцију интерферона-гама, и показали да је и његова продукција смањена у ко-култивацији bEnd.3 ћелија и ћелија лимфног чвора. Запажена различитост у деловању ћелија можданог ендотела на два основна проинфламаторна цитокина је од значаја за разумевање комплексног процеса регулације инфламације у CNS.