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Lymphocyte migration through cultured endothelial cell monolayers derived from the blood–retinal barrier

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SUMMARY

Lymphocyte migration across endothelial monolayers, derived from the rat blood–retinal barrier, was recorded *in vitro* using time-lapse video microscopy. Syngeneic lymphocytes were plated out onto endothelial cell monolayers for 4 hr and their surface motility and transmonolayer migration recorded and quantified. Under resting conditions lymphocytes, obtained from peripheral lymph nodes (PLN), were small, rounded and static with less than 5% migrating across the monolayer. Activation of the lymphocytes with concanavalin A (Con A) increased their size and surface motility on both interferon-γ (IFN-γ)-treated and resting endothelia, but did not alter the number migrating across the monolayer. Similar results were also found for phytohaemagglutinin (PHA)-activated lymphocytes. Interleukin-2 (IL-2)-dependent CD4$^+$ T-cell lines specifically recognizing either retinal soluble antigen (S-Ag) or bovine serum albumin (BSA) exhibited significantly greater surface motility over the endothelial monolayers than the mitogen-activated PLN lymphocytes. By 4 hr, in excess of 50% of the T-cell line lymphocytes had migrated across the endothelial monolayer. Treatment of the endothelial cells with IFN-γ caused a small, but not significant, increase in the level of T-cell line lymphocyte migration. These results suggest that the migration of lymphocytes across central nervous system-derived endothelia is primarily dependent upon the state and mode of lymphocyte activation.

INTRODUCTION

Lymphocyte migration across the vascular bed of an organ plays a central role in the development of many chronic inflammatory disorders. This complex event is believed to be controlled by interactions between lymphocytes and vascular endothelia via a group of surface receptors.[1] During the different stages of lymphocyte extravasation, independent receptor pairings have been shown to dominate,[2] in particular during the rolling, adhesive and migratory steps. These processes, however, are not static and are subject to alteration according to the state of activation of both the lymphocyte and the endothelia.[3,4] Furthermore, it has been demonstrated that these associations are not uniform throughout the vasculature but differ between endothelia from various tissues. It is well recognized that endothelia from the central nervous system (CNS) differ from those outside the CNS in that they form a highly selective blood–tissue interface. One consequence of the blood–brain and blood–retinal barriers (BBB and BRB, respectively) is the concept that the CNS is an immune privileged site. Migration of activated lymphocytes into normal CNS can occur,[5] although it is only during chronic inflammatory disorders of the CNS, such as multiple sclerosis and posterior uveitis, that the degree of lymphocyte infiltration becomes considerably up-regulated.

The adhesive interactions that occur during the initial stages of lymphocyte extravasation have been investigated extensively. This has led to a clearer understanding both of the kinetics of adhesion and of the adhesion molecules involved. The ensuing process of migration through the vessel wall, however, is far less well understood. In recent *in vitro* studies the migration of lymphocytes across non-CNS vasculature has been reported to be mediated in part by the adhesion molecules LFA-1 and ICAM-1.[2,6,7] This process can be readily up-regulated by activation of the endothelium with interferon-γ (IFN-γ).[8,9] Lymphocyte migration across CNS endothelia, however, remains to be elucidated. Studies *in vitro* have shown that lymphocyte adhesion to brain[10,11] and retinal[12] endothelia is significantly less than to non-CNS endothelia, but whether differences also exist in the migratory process between endothelia is unclear.

In this study we have investigated the kinetics of lymphocyte migration through retinal endothelial cell (REC) monolayers, employing a rat REC culture as an *in vitro* model of the retinal vascular barrier. By altering the state and mode of activation of lymphocytes we have explored the effect of these treatments upon lymphocyte motility and migration. In addition, the

Abbreviations: CNS, central nervous sytem; PLN, peripheral lymph node; REC, retinal endothelial cell; S-Ag, retinal soluble antigen.

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consequence of treating the REC monolayer with IFN-γ upon this process was also evaluated.

MATERIALS AND METHODS

Reagents

Rat recombinant IFN-γ was obtained from Holland Biotechnology bv (Leiden, The Netherlands) and purified recombinant interleukin-2 (IL-2) and collagenase/dispase from Boehringer Mannheim (Lewes, U.K.). Hanks' balanced salt solution (HBSS) and plastic culture dishes were from Gibco (Paisley, U.K.). Lymphocytes were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco) and 5×10^{-5} M 2-mercaptoethanol (Sigma, Poole, U.K.). Concanavalin A (Con A; Type V), phytohaemagglutinin (PHA), Percoll, collagen (type 1) and bovine serum albumin (BSA) were all from Sigma.

Endothelial cells

Specific pathogen-free female Lewis rats (50–120 g; Harlan Olac Ltd, Bicester, U.K.) were used throughout. Retinal microvessels were isolated and primary cultures of endothelial cells were grown to confluency according to a previously described method.[13] Briefly, retinae were removed from the eyes of young female Lewis rats and digested in collagenase/dispase for 1 hr. Microvessel fragments were isolated from contaminating single cells by density centrifugation, further enzymatic digestion and finally by separation on a 50% pre-formed Percoll gradient. The microvascular fragments were finally suspended in culture medium as previously described[13] and plated out onto collagen-coated plastic culture plates. The endothelial cells were allowed to grow to near confluence over a 2–3-week period in 5% CO_2 at 37°, with the culture medium being replaced every 2–3 days. Primary cultures were used throughout.

Lymphocytes

Lymphocytes were obtained from peripheral lymph nodes (PLN) of female Lewis rats as previously described.[11] Terminally differentiated IL-2-dependent $CD4^+$ T-cell lines specifically recognizing retinal soluble antigen (S-Ag) were established and maintained by weekly restimulations with S-Ag (10 μg/ml) using 50-fold irradiated autologous thymocytes as feeder cells.[14,15] Cells were grown at 2×10^5/ml in the presence of purified recombinant IL-2 and restimulated with antigen prior to use. A non-ocular antigen-specific T-cell line was established from BSA-primed syngeneic animals. The T cells were stimulated with BSA (10 μg/ml) and a 50-fold concentration of irradiated autologous thymocytes. Cells were grown in the presence of IL-2 and restimulated with antigen as described above prior to use.

Lymphocyte and endothelial cell activation

PLN cells were activated by incubating with either Con A (5 μg/ml) for two separate time periods of either 24–48 hr or 48–72 hr, or alternatively with phytohaemagglutinin (PHA; 1 μg/ml) for between 48 and 72 hr. Prior to the migration assay the PLN cells were washed thoroughly with HBSS to remove any residual mitogen. Retinal endothelial cell monolayers were activated with IFN-γ (100 U/ml) 18 hr prior to the migration assay as this dose and duration have previously been shown to produce maximal adhesion.[11]

Migration assay

PLN cells or antigen-specific T cells (6×10^5 cells/well, 2×10^5 cells/ml) suspended in culture medium were added to REC monolayers and placed onto the stage of an inverse-phase contrast microscope housed in a temperature controlled (37°) environment. A field of 200 μm^2 was selected and continuously recorded using a video camera attached to the microscope and stored on a time-lapse video tape recorder. After 4 hr of co-culture the recording was stopped, replayed at 160 × normal speed and analysed by enumerating, at 30-min intervals, the number of cells within the field that were (1) static, (2) spread and motile on the surface of the REC monolayer, and (3) motile underneath the monolayer between the culture dish and the underside of the endothelia, the latter group having migrated across the monolayer.

The migrated lymphocyte population could be clearly distinguished from those remaining on the surface by their distinctive morphology and refractive appearance, characteristics previously used to identify granulocytes that had migrated across aortic endothelial monolayers[16,17] and lymphocytes across high endothelial cell monolayers.[18] Motility was defined as those lymphocytes that exhibited pseudopodia and actively moved over or under the REC monolayer during an 8-min time period spanning each 30-min time-point. The data were then expressed as the percentage of lymphocytes within the field that were motile and the percentage of lymphocytes that had migrated underneath the monolayer. All values are given as means ± SEM and differences between groups were assessed using the non-parametric Mann–Whitney *U*-test.

RESULTS

Phase-contrast appearance

By replaying the time-lapse video at 160 times the original speed, the lymphocytes moving over the surface of the REC monolayer were readily distinguishable from those underneath the monolayer by their phase-contrast appearance and morphology. Lymphocytes on the surface were highly refractive and were either rounded or partially spread. In contrast, those underneath the monolayer were phase dark and very spread (Fig. 1) and moved with a distinctive probing motion. The presence of lymphocytes underneath the monolayer has been confirmed with transmission electron microscopy (unpublished data). Lymphocytes could be observed migrating through the monolayer, although the degree of resolution did not allow us to determine whether this occurred at the cell junction or close to it. Once underneath the monolayer all lymphocytes remained motile.

Migration of PLN cells

When resting PLN cells were added to endothelial cell monolayers only 0·8 ± 0·4% of the cells in the field were found to have migrated through the monolayer by 4 hr (Fig. 2). Following activation of the PLN cells with Con A for either 24–48 hr or 48–72 hr there was no increase in the percentage of cells migrating through a resting endothelial cell monolayer. Similarly, when

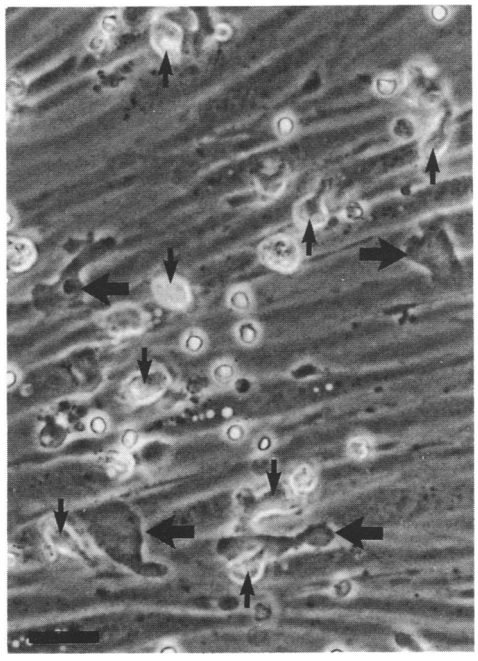

Figure 1. Inverse-phase contrast photomicrograph of co-culture of resting REC monolayer after 2 hr with S-Ag T-cell line lymphocytes. Lymphocytes on the surface of the REC monolayer are highly refractive and more rounded (small arrows) whilst those that have migrated through and lie between the underside of the monolayer and the plastic culture dish are flattened and phase dark (large arrows). Bar = 20 μm.

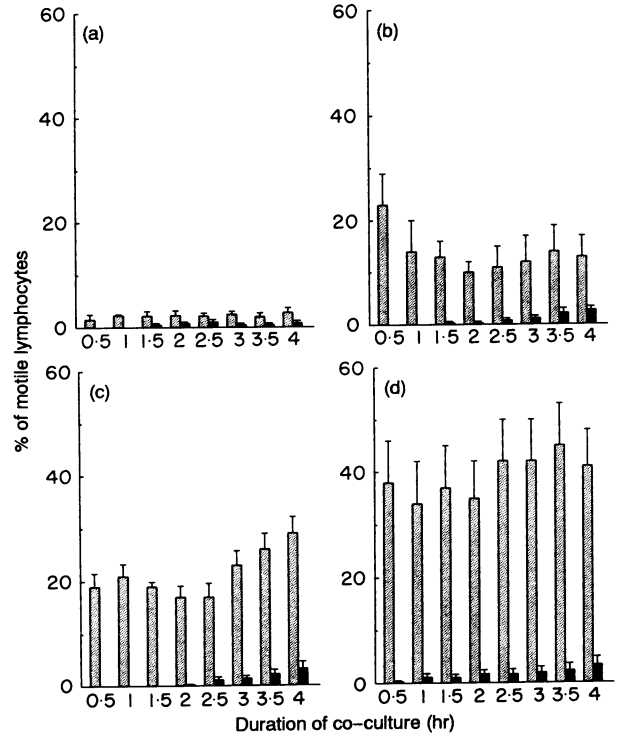

Figure 3. Percentage of motile lymphocytes within field above (▨) and below (■) REC monolayer. (a) Resting PLN cells and REC; (b) 24–48 hr Con A-activated PLN cells and IFN-γ-activated REC; (c) 48–72 hr Con A-activated PLN cells and resting REC; (d) 48–72 hr Con A-activated PLN cells and IFN-γ-activated REC. Values are means ± SEM of a minimum of six separate experiments.

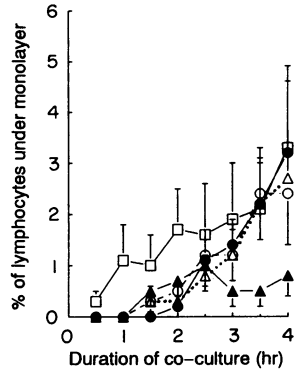

Figure 2. Migration of PLN lymphocytes across REC monolayers under different conditions of cell activation. (▲——▲) Resting PLN cells and REC; (●——●) 48–72 hr Con A-activated PLN cells and resting REC; (△. . . .△) 24–48 hr Con A-activated PLN cells and IFN-γ-activated REC; (□——□) 48–72 hr Con A-activated PLN cells and IFN-γ-activated REC; (○- - -○) 48–72 hr PHA-activated PLN cells and IFN-γ-activated REC. Values are means ± SEM of a minimum of six separate experiments.

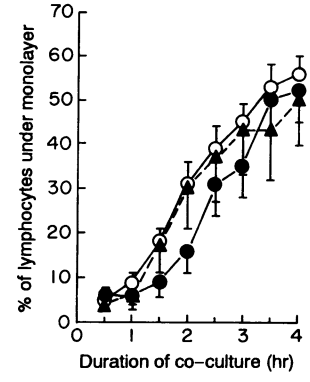

Figure 4. Migration of antigen-specific T-cell line lymphocytes across REC monolayers. (●——●) S-Ag T-cell line on resting REC monolayers; (▲- -▲) BSA T-cell line on IFN-γ-activated REC; (○——○) S-Ag T-cell line on IFN-γ-activated REC. Values are means ± SEM of a minimum of six separate experiments.

PLN cells were stimulated with PHA for 48–72 hr and co-cultured with IFN-γ-activated endothelium there was no effect on migration (Fig. 2).

When PLN cells activated with Con A for 48–72 hr were cultured with IFN-γ-stimulated endothelia the percentage of cells migrating across the monolayer was slightly increased (Fig. 2) but did not reach significance at any time-point.

Motility of PLN cells

Resting PLN cells in co-culture with resting REC monolayers remained small, static and rounded with only occasional evidence of filopodia. Under these conditions a total of only 3·6 ± 0·8% exhibited motile activity above and below the monolayer by 4 hr (Fig. 3a). Mitogen activation significantly

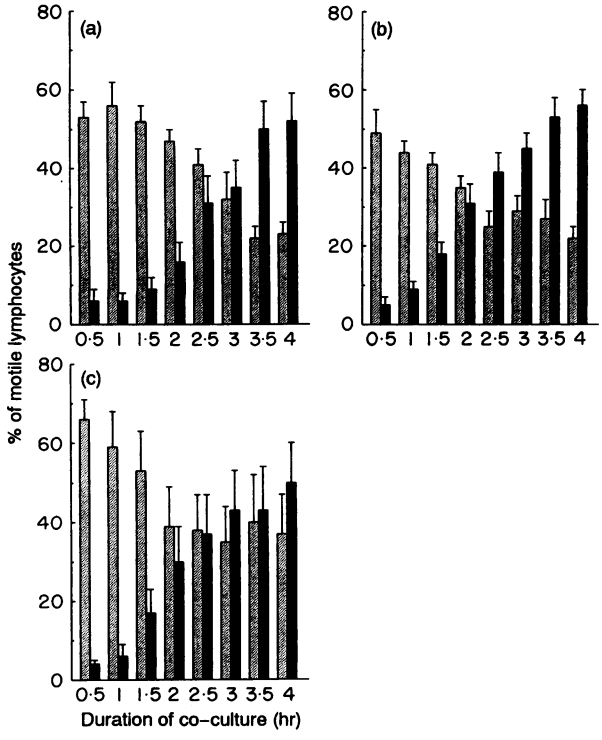

Figure 5. Percentage of motile lymphocytes within field above (▨) and below (■) REC monolayer. (a) S-Ag T-cell line on resting REC monolayer; (b) S-Ag T-cell line on IFN-γ-activated REC; (c) BSA T-cell line on IFN-γ-activated REC. Values are means ± SEM of a minimum of six separate experiments.

increased the degree of PLN cell motility in all cases. Following Con A activation of the PLN cells for 24–48 hr and IFN-γ activation of the endothelium, 16 ± 3% of PLN cells demonstrated motile activity at 4 hr (Fig. 3b). By activating the PLN cells with Con A for 48–72 hr they became larger and exhibited more filopodia. After 4 hr of co-culture with resting endothelia, the level of motility was increased to 32 ± 3% (Fig. 3c). This level could be further increased to 44 ± 7% upon IFN-γ activation of the monolayer (Fig. 3d) which approached the degree of motility observed with the T-cell lines. In all cases, however, PLN cell motility was predominantly restricted to the surface of the retinal endothelial cell monolayer. With PHA activation the degree of motility of the PLN cells was less than that of Con A-activated cells. Activation of the REC monolayer with IFN-γ increased the number of motile PLN cells (Fig. 3c and d), although there was no corresponding increase in migration through the monolayer.

Migration of T-cell lines

The migration of antigen-specific T-cell line cells through REC monolayers was significantly greater than with the PLN cells. During the 4-hr co-culture, T cells could be seen migrating through the monolayer. Although occasionally lymphocytes could be seen to migrate from underneath the monolayer back onto the surface, there was a net increase in the number of lymphocytes underneath during the assay. By 4 hr the percentage of S-Ag T-cell lymphocytes under the endothelial monolayer was 52 ± 7% on resting endothelium and 56 ± 4% on IFN-γ-activated endothelium (Fig. 4). With the BSA T-cell line 50 ± 10% T cells had migrated through the activated endothelial cell monolayer by 4 hr. After the first half hour of co-culture, there was a consistently greater migration of S-Ag T-line cells through the IFN-γ-activated monolayer than with the non-activated endothelia, although this trend proved not to be statistically significant.

Motility of T-cell lines

The antigen-specific T-cell lines were generally much larger, spread and motile than the activated PLN cells. The T-cell lines displayed a significantly higher degree of motility after 4 hr of co-culture than the mitogen-stimulated PLN cells. The percentage of motile S-Ag-specific lymphocytes on the surface of the endothelial monolayer after 30 min was not significantly different to the motile population of 48–72 hr Con A-activated PLN cells on activated endothelium, although with the BSA T-cell line there was a significant difference. However, with the T-cell lines the number of cells exhibiting motile behaviour on the monolayer surface decreased with time as the percentage migrating through the monolayer increased (Fig. 5). The total number of motile cells (i.e. above and below the monolayer) also increased with time, reaching a value of 75 ± 5% (S-Ag T-cell line on resting endothelia), 78 ± 4% (S-Ag T-cell line on activated endothelia) and 87 ± 3% (BSA T-cell line on activated endothelia) of the total lymphocytes in the field by 4 hr.

DISCUSSION

The initial stage in the process of lymphocyte migration across retinal endothelium is thought to involve adhesive interactions between the endothelial cell and lymphocytes. In a separate study we have shown that lymphocytes bind to cultured rat retinal endothelia to a much lower degree than to peripheral endothelia[12] but in a manner similar to that described for brain endothelia.[10,11] The subsequent process of migration across either brain or retinal monolayers has not previously, to our knowledge, been described in detail, although preliminary data on migration across brain endothelium *in vitro* have been reported.[19,20] However, in studies in which migration across non-CNS endothelia has been explored it is clear that the migratory process is controlled by cell-surface receptors which may be induced following cell activation.

The migration of T cells in this study can be interpreted in the absence of any influence from chemotactic gradients existing across the REC monolayer. By activating lymphocytes with antigen we have demonstrated that the proportion of cells migrating across a REC monolayer *in vitro* can be significantly up-regulated when compared to resting or mitogen-activated PLN lymphocytes. Although mitogen activation of lymphocytes and IFN-γ activation of endothelial cells have previously been shown to increase lymphocyte adhesion to brain[10,11] and retinal endothelia,[12] they did not significantly increase lymphocyte migration through the REC monolayer despite increasing cell motility. The process of migration through the vascular endothelial barrier of non-CNS tissue is thought to be mediated to a large extent by the LFA-1/ICAM-1 receptor pairing which is only marginally involved in adhesion.[2,6,7] Activation of endothelial cells with IFN-γ has been shown to induce an increased expression of ICAM-1.[21] This increase may account

for the concomitant increase in migration that has been reported in non-CNS endothelia both *in vitro*[9] and *in vivo*[8,22] following activation with this cytokine. IFN-γ also increases the expression of ICAM-1 on retinal endothelium,[23] although we were unable to demonstrate a statistically significant increase in the level of migration across REC monolayers following IFN-γ treatment. Although there was no significant difference in the migration of the S-Ag T-cell line between resting and IFN-γ activated REC, there were consistently more lymphocytes under the monolayer in the IFN-γ-activated group. It is possible, however, that these findings reflect a genuine difference between CNS and non-CNS endothelia. Investigations are currently underway to elucidate the effects of other cytokines on lymphocyte migration and the specific receptor pairings that mediate this process.

The extremely low level of PLN cell migration through the REC monolayer is consistent with the *in vivo* studies of Issekutz.[22] Activation of PLN cells with Con A did marginally increase the degree of migration after 48 hr of activation but this did not reach significance. It is unlikely that the low level of migration is due to lectin-specific binding causing retention of the cells on the surface, as previous studies have shown that Con A-treated red blood cells under identical conditions do not bind to endothelial monolayers.[11] In addition, we have found that Con A treatment of the S-Ag T-cell line does not reduce migration (unpublished data). Although Con A failed to enhance migration we have shown in a complementary study that the low level of PLN cell adhesion to REC monolayers could be increased significantly following Con A activation and that this effect was maximal by 6 hr.[12] As with the T-cell line studies, IFN-γ activation of REC did not increase PLN cell migration to a significant degree but did increase the level of lymphocyte motility. This enhanced motility may result from an increase in the expression of ICAM-1 as the LFA-1/ICAM-1 pairing has been implicated in cell locomotion.[6]

Both the S-Ag and BSA T-cell lines exhibited considerable migratory behaviour with in excess of 50% of the cells migrating through the REC monolayer and remaining underneath by 4 hr. These findings are consistent with the *in vivo* studies of Hickey *et al.*[5] in which T-cell lines raised against either a CNS-derived or a non-relevant antigen migrated into the brain equally. Although there are no equivalent markers in the rat for the so-called human memory ($CD45RO^+CD29^+$) and naive ($CD45RA^+$) T cells, it is likely that these T-cell lines are memory cells due to their having been restimulated with antigen several times. Indeed, they have been shown to be $OX22^-$ (unpublished results), which corresponds with the memory phenotype in man,[24] and no differences were found in migration between cell lines of different passages. The ability of these cells to migrate through the monolayer, therefore, supports earlier human *in vivo* studies in which preferential migration of $CD45RO^+CD29^+$ cells over $CD45RA^+$ cells was recorded.[25] Consistent with this is the observation that human memory T cells express enhanced levels of LFA-1,[26] and that antigen activation increases LFA-1 avidity.[27] Since the ligand pairing of LFA-1/ICAM-1 is critical in lymphocyte migration across non-CNS vasculature[2,6,7] it is likely that this pairing is also involved in the migration of rat T-cell lines across CNS-derived REC monolayers.

If we are to understand the immunopathogenesis of putative autoimmune T-cell-mediated diseases such as multiple sclerosis and posterior uveitis and develop effective immunotherapeutic strategies, it is essential that the mechanisms by which lymphocytes enter the CNS under both normal and pathogenic conditions are clearly defined. How the disease is initiated and perpetuated is likely to depend upon the ability of immune cells to enter the tissue and for antigen presentation to occur. In this study we have demonstrated that antigen-specific terminally differentiated $CD4^+$ T cells readily migrate across CNS-derived REC monolayers but that lymphocytes obtained from peripheral lymph nodes, whether mitogen-activated or not, are less able to cross the CNS vascular barrier. To further determine the mechanisms behind these functional differences we are currently investigating the effects of other cytokines on migration and the differential role played by the adhesion molecules in this process. In addition, it has recently been shown that REC express both MHC class I and class II molecules under appropriate conditions[28] and the ability of these cells of the BRB to present antigen is being studied.

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