Microglia Induce CD4 T Lymphocyte Final Effector Function and Death

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Summary

Microglia, a type of tissue macrophage, are the only cells in the central nervous system (CNS) parenchyma to express some major histocompatibility complex (MHC) class II constitutively or to upregulate expression readily. They are thought to play a role in CD4 T cell activation in autoimmune diseases such as multiple sclerosis, as well as in neurodegenerative conditions, Alzheimer's disease in particular. We show here that highly purified MHC class II+ microglia when tested directly ex vivo do indeed support an effector response by an encephalitogenic myelin basic protein-reactive CD4 T cell line from which production of the proinflammatory cytokines, interferon-γ and tumor necrosis factor, is elicited, but not interleukin (IL)-2 secretion or proliferation. After this interaction, the T cells die by apoptosis. Other nonmicroglial but CNS-associated macrophages isolated in parallel stimulate full T cell activation, including IL-2 production, proliferation, and support T cell survival. Neither CNS-derived population expresses B7.1/B7.2. Resident macrophages that terminate effector T cells in tissues constitute a novel and broadly applicable regulatory measure of particular relevance to processes of self-tolerance against sequestered antigens.

All tissues, including those previously believed to be privileged sites such as the central nervous system (CNS)

1 Abbreviations used in this paper: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MG, microglial cells.

dence cited in support of the proposal that such diseases may have an underlying immunological/inflammatory pathogenesis. A synergistic interaction between β-amyloid protein, IFN-γ (predominantly a T cell cytokine), and MG, in induction of neuronal cell damage in vitro, has also been reported (9). Nevertheless, the argument that MG are an important stimulator of both primary and secondary T cell responses in the CNS and the experimental evidence in support of this concept (10, 11), is inconsistent with a number of pieces of experimental data, namely: (a) The observed paucity of activated or proliferating T cells in the parenchyma of inflamed CNS (12, 13) in which a network of MHC class II+ MG can normally be found; (b) MG constitutively express MHC class II most highly in the EAE-resistant Brown Norway rat strain (8); and (c) intra-CNS administration of IFN-γ, while strongly upregulating MG MHC class II expression, inhibits or reduces the severity of EAE (14), while anti-IFN-γ mAb treatment enhances EAE severity (15).

To resolve this issue, activated MG were purified by flow cytometric sorting from the adult CNS (16, 17) to enable immediate ex vivo analysis of the APC role of MHC class II+ MG for myelin basic protein (MBP)-reactive CD4 T cell responses. We show here that MG support only an incomplete form of T cell activation and induce apoptotic death of the responding T cells.
A Normal GVHD

CD11 b/c

MHC class II

CD45

B

CNS MΦ
Pop. 2

Blood vessel

MG Pop. 1

CNS parenchyma

C Normal microglia (Population 1) Normal CNS MΦ (Population 2)

D 12-14 hrs 48 hrs 5 days

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Materials and Methods

Antibodies and Flow Cytometry. Mouse mAb for flow cytometric sorting and analysis were MRQOX1 (anti-CD45 morphologic), MRQOX6 (anti-MHC class II), MRQOX42 (anti-CD11b and c), R73 (anti-a/β TCR), W3/25 (anti-CD4), MRQOX12 (anti-rat Ig κ chain), MRQOX39 (anti-CD25), and MRQOX40 (OX40 antigen (TNFR/nerve growth factor receptor family), MRQOX21 (mouse anti-human C3b and not rat cells), K1-21 (mouse anti-human free κ-light chain non-cross-reactive with rat Ig) and mouse anti-human CD4-PE (Sero- tec, Oxford, UK) served as negative controls. See (12, 18-21) for reference or cross-reference details. PE-conjugated OX1 (anti-CD45 and R73 (anti-α/β TCR) were from Pharmingen, San Diego, CA. For direct staining, mAb were purified, then conjugated to either FITC or biotin. Biotinylated mAb were detected with either streptavidin-PE (two-color flow cytometry; Caltag Laboratories, San Francisco, CA) or streptavidin-allophycocyanin (three-color flow cytometry; Molecular Probes, Eugene, OR).

Flow cytometry in which mouse mAb tissue culture supernatants were used, mAb were detected with rat-absorbed FITC-conjugated sheep F(ab')2, anti-mouse Ig (Sigma Chemical Co., St. Louis, MO). CTLA-4-Ig derived from the hybridoma cell line J558L transfected with the mouse CTLA-4/human IgG1 fusion protein DNA construct (22), was isolated from culture supernatant by protein G-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography, and biotinylated. Two-color flow cytometric analysis was performed on a FACSscan® (Becton Dickinson and Co., San Jose, CA) while three-color analysis or sorting was executed with a dual-laser FACStar®PLUS® (Becton Dickinson and Co.).

GVHD Induction and Cell Isolation. GVHD was induced in 2 Gy γ-irradiated adult brown Norway × Lewis strain (FI) rats by injection i.v. of 2 × 10⁶ Lewis strain splenocytes. 10 d after splenocyte injection, animals were killed, perfused extensively, the CNS removed from the GVHD-affected as well as from normal FI rats, and MG and CNS-associated macrophages/leukocytes purified to homogeneity, as described (17).

Assays for T Cell Activation and Apoptosis For each experiment, MG from day-10 GVHD FI rats and CD45⁺/mph macrophages from the normal FI rat CNS were isolated by staining and flow cytometric sorting from two groups of four to six perfused rats. Post-sort purities were always in excess of 95%. Average yields (per rat) of viable cells after sorting were 3.5 × 10⁶ GVHD-activated microglia and 4 × 10⁶ normal CNS macrophages. These CNS APC were resuspended in RPMI 1640–5% FCS and added immediately to wells of a 96-well U-bottom microtiter plate. Cells were used on the day of isolation and never precultured before use. Results were identical whether CNS APC were irradiated (20 Gy) before culture with T cells, or not. Normal LN cells as control APC were isolated from FI rats, and irradiated (20 Gy). Lewis rat strain encephalitogenic (EAE-inducing) MBP-specific or OVA-specific CD4 T cell lines (23) were added to CNS-derived or LN APC together with either whole purified guinea pig MBP (15 µg/ml final concentration) ± IL-2 (saturation concentrations), the 68–86 encephalitogenic peptide of MBP (5 µg/ml), OVA (40 µg/ml), or no antigen. Subsequent treatments were: (a) At 24 h, supernatants were taken for cytokine analysis. Cytokine assays were: TNF - WEHI 164 cell cytotoxicity (24); IL-2-CTL bioassay (25); IFN-γ-rat IFN-γ ELISA (Gibco BRL, Gaithersburg, MD). Units were determined by titration against recombinant cytokines in each case. (b) The cultures were left for 48 h and pulsed with 0.5 µCi [3H]thymidine/well for harvest 14 h later and measurement of proliferation (cpm). Qualitatively similar results were obtained using culture times of 60 h + 14 h [3H]thymidine pulse. (c) At 24 h, cells were recovered from the wells, washed, recultured for a further 24 h in RPMI 1640–5% FCS ± IL-2, then fixed, permeabilized and double-stained for α/β TCR, and for apoptotic cells (FITC-DUTP) by the TUNEL technique (Cell Death Detection Kit; Boehringer Mannheim GmbH, Mannheim, Germany). For estimation of apoptosis by flow cytometry, a live scatter gate was applied to reduce the contribution to analysis, of any TUNEL-positive cells dying by necrosis. (d) At 24 h, cells were recovered from the wells, washed, and recultured for a further 48 h with IL-2. These partially rested cells were added to wells coated with anti-α/β TCR mAb or an isotype-matched control mAb, and proliferative responses assessed by [3H]thymidine incorporation after 48 h total incubation time.

In Vitro B Cell Activation. Flat-bottom 96-well tissue culture plates (Costar Corp., Cambridge, MA) were coated with rat Ig absorbed rabbit anti–mouse IgG by addition of 50 µl/well antibody (100 µg/ml in PBS) and overnight incubation at 4°C. Wells were washed three times with PBS and 50 µl/well mouse mAb tissue culture supernatant specific for rat Ig κ chain added for 1–2 h at 37°C. The anti-rat Ig-coated wells were washed thoroughly with PBS and dissociated rat spleen cells in RPMI 1640 (Cyto- systems, Sydney, Australia)–10% FCS (Trace, Sydney, Australia) added and cultured for a total of 48 h. B cell blasts were apparent within 24 h. Cells were recovered from the culture plates and stained for flow cytometry.

Microglial Cell Culture. Highly purified MG from GVHD-affected rat CNS were cultured on tissue culture grade plastic (Costar Corp.), in RPMI 1640–5% FCS. Cultures were maintained in a 5% CO₂ atmosphere at 37°C.

Results and Discussion

Flow Cytometric Purification of Microglia and CD45⁺/mph CNS Macrophages. Macrophage lineage cells in the adult CNS consisting of intraparenchymal long-lived, irradiation-resistant fixed-tissue macrophages (or microglia, Fig. 1, A and B, CD45⁺/mphCD11b/c⁺, population 1) (26) and transient, vessel-associated blood-derived macrophages (Fig. 1, A and B, CD45⁺/mphCD11b/c⁺, population 2), were separated on the basis of CD45 expression levels (16). The distinct nature and anatomical location of these populations have been demonstrated in irradiation bone marrow chimeras in which substantial turnover only of the CD45⁺/mphCD11b/c⁺ (non-MG) population from blood-derived precursors is ob-

Figure 1. Flow cytometric separation of CNS macrophage-lineage cells. (A) Flow cytometric plots of MG/leukocyte-enriched populations from the CNS of normal healthy control rats or rats with GVHD at day 10. (B) Intraparenchymal MG (population 1) are separated anatomically from blood-derived macrophages (population 2). (C) May-Grünwald-Giemsa-stained cytospins of these populations isolated directly from the normal CNS by flow cytometric sorting using the well-separated sorting gates 1 and 2. (D) Freshly isolated GVHD-activated MG cultured for the times shown. (Arrow) Spiked processes typical for MG. Phase contrast microscopy. Bars: (G) 10 µm; (D) 15 µm.
T cells alone

T cells + Microglia

T cells + Microglia + Antigen

Figure 2. T cell activation with microglial cell APC. Surface phenotype of MBP-reactive CD4 T lymphocytes after MG culture. MBP-specific CD4 T cell lines were cultured alone, or together with MG purified from day 10 GVHD F1 rats (1:1 ratio), for 24 h with or without added MBP. Cells were recovered from the culture wells and dual labeled for CD11b/c and either α/β TCR, CD4, CD25, or MRC OX40. The phenotype only of the CD4 T cell lines added to culture was assessed by electronically gating-out CD11b/c + cells (MG). For forward scatter, dotted lines in the middle and lower boxes represent the profile of T cells cultured alone. Other histograms illustrate the surface antigen expression of the T cells (solid histograms, mean fluorescence indicated) and autofluorescence of the same cells stained with isotype-matched control mAb (dotted lines).

served (17, 27). Cytologically, the two populations were quite distinct on isolation (Fig. 1 C). MG exhibited a typical heterochromatic nucleus and lacked the cytoplasmic granules of conventional CNS macrophages.

In GVHD, T cell blasts but few if any blood monocytes enter the CNS (28) in a manner more reminiscent of normal T cell patrolling of the CNS than inflammatory CNS disease like EAE. By flow cytometric sorting and immunohistological analysis of MG and CNS tissue from rats with GVHD we have shown that MG are activated directly by these infiltrating T cells (Ford, A.L., E. Foulcher, and J.D. Sedgwick, manuscript submitted for publication). MG increased in number by up to six times, their levels of CD45 expression were raised three- to fourfold (Fig. 1 A, population 3) and all MG up-regulated MHC class II (Fig. 1 A). CD45high CD11b/c+ cells (population 2) were not clearly defined in the GVHD CNS and were reduced numerically, possibly reflecting the radiation of the recipient preparatory to induction of GVHD. The relative lack of inflammatory macrophages permitted a clear distinction to be made between activated MG (Fig. 1 A, sort gate 3) and other CNS macrophages. MG sorted from the GVHD-affected CNS were comparable morphologically to those obtained from the normal CNS (Fig. 1 C) apart from an increase in cell volume of ~10%. Unlike MG from the normal CNS (17), GVHD-activated MG, when cultured, adhered to plastic within a few hours, developed many processes, and became highly motile (Fig. 1 D). The cells also phagocyted 1 μm latex beads (Ford, A.L., E. Foulcher, and J.D. Sedgwick, manuscript submitted for publication).

Microglia Support an Incomplete Form of T Cell Activation.

The APC potential of MG was examined by culturing MBP-reactive CD4 T cells in the presence of activated freshly isolated MHC class II+ MG. A rapid antigen-specific increase in the size of T cells from MG/T cell cultures containing MBP was detected both microscopically and flow cytometrically in the form of a shift in forward light scatter (Fig. 2). This was accompanied by phenotypic changes in the T cells, with some reduction in α/β TCR and CD4 expression and substantial up-regulation of CD25 (IL-2R α-chain) and OX40 antigen expression. The OX40 antigen is up-regulated upon CD4 T cell activation (20). The expression of CD25 and MRC OX40 on T cells cultured alone reflects some residual expression of these antigens and is typical for CD4 T cell lines in culture. The minor down-regulation of α/β TCR expression and threefold increase in OX40 expression obtained in the absence of antigen (Fig. 2) may indicate some level of constitutive MBP presentation to T cells. Activated microglia are known to phagocytose myelin in vivo (29).

Consistent with previous studies on the role of non-MG CNS macrophages in antigen presentation both in vivo (30) and in vitro (17), CD45high macrophages from the normal CNS supported vigorous antigen-specific T cell prolif-
The definition of units for each antigen was significantly greater than that of all other controls without antigen. Similar experiments. *Proliferation of cells in these cultures without antigen, proliferated normally relative to culture in fresh medium (not shown). Despite the failure to induce significant T cell proliferation, antigen-specific induction of both TNF and IFN-γ (Fig. 3 B) occurred in the presence of MG at levels comparable to that produced by T cells cultured with CD45+ CNS-derived macrophages. The most profound difference in terms of cytokine production was the lack of detectable IL-2 in supernatants from MG/T cell cultures (Fig. 3 B).

These experiments show that antigen presentation by MG produces an incomplete form of T cell activation, without IL-2 secretion or proliferation but with proinflammatory cytokine production enabled. The inability of MG to support proliferation after the addition of exogenous IL-2 distinguishes this process from IL-2 reversible T cell anergy seen after antigen presentation in the absence of appropriate costimulation (31). The process is not one of immune deviation (32), involving as it does, MG interaction with an already fully differentiated type 1 CD4+ effector T cell which, other than loss of IL-2 production, continues to exhibit type 1 cytokine characteristics in that secretion of TNF and IFN-γ is preserved (Fig. 3 B). The inability of MG/T cell-conditioned supernatant to inhibit T cell proliferation provides no evidence for the role of secreted factors in mediating T cell unresponsiveness. Thus it is likely that the direct MG–T cell interactions are responsible.

Microglia Induce T Cell Apoptosis. To study the downstream consequences of T cell–MG interaction, T cells were stimulated with antigen for 24 h in the presence of MG and cultured for a further 24 h in medium (Fig 4 A, I). In these cultures, apoptosis was detected in ~20% of the T cell population. Apoptotic T cells expressed lower levels of α/β TCR. By contrast, no apoptotic changes were observed in T cells that were cultured alone or in the presence of other APC. T cells cultured together with CNS macrophages and antigen at the same ratio (1:1) as used in the MG cultures underwent full T cell activation (Fig. 3) but no apoptosis (Fig. 4 A, I). These results therefore demonstrate that it was the MG and not simply the induction of T cell activation (producing, for example, T cell–T cell-mediated apoptosis [33]), that was important in this process.

Addition of IL-2 for 24 h after culture with MG (Fig. 4 A, I) resulted in almost complete rescue of T cells from MG-induced apoptotic death. As noted earlier (Fig. 3 A), addition of exogenous IL-2 to MG/T cell cultures did not promote proliferation of T cells despite the fact that they expressed the IL-2 receptor at high levels (Fig. 2). The ability of exogenous IL-2 to act as a survival factor for T cells after culture with MG effectively excludes the possibility that the expressed IL-2 receptor was nonfunctional. Thus, this mechanism differs from the reversible state of T cell unresponsiveness induced by the interaction of lung macrophages with activated T cells in which IL-2 production was reported to be maintained but signal transduction via the IL-2 receptor was defective (34).

Apoptosis appeared to be antigen dependent since its incidence was much lower in MG/T cell cultures lacking antigen (Fig. 4 A, III, 5.4% vs 19.7%). The residual level of apoptosis seen in the absence of added antigen was presum-
Figure 4. Microglial APC but not other CNS macrophages induce IL-2–reversible apoptosis and anergy in CD4 T cells (A) Antigen-specific apoptosis in CD4 T lymphocytes cultured in the presence of MG. Culture conditions were as per Fig. 3. Numbers are percent TUNEL–positive α/βTCR+ cells. Quadrants defining FL-2 (y-axis) autofluorescence and TUNEL background levels were determined by staining cells with isotype-matched control mAb and FITC-dUTP, but in the absence of terminal deoxynucleotidyl transferase, respectively. (B) Proliferative responses of MBP or OVA–specific CD4 T lymphocyte lines to TCR cross-linking after MG or LN APC culture (+ specific antigen) and 48-h IL-2 rescue (as per group II cells in A but with an additional 24 h of IL-2). Bars represent means of triplicate determinations. Magnitude of T cell proliferation on immobilized α/βTCR mAb of cells preexposed to LN APC vs MG, was significantly less in each case (OVA 1, P < 0.05, MBP 2, P < 0.01, paired t test).

IL-2–rescued T Cells Fail to Respond to TCR Cross-Linking. IL-2 is a recognized T cell survival factor (35) that, particularly in secondary T cell responses, is associated with increased production of Bcl-xL and Bcl-2 survival proteins (36). The capacity of IL-2 to rescue T cells from apoptosis (Fig. 4 A, I) has important implications for the pathogenesis of CNS disease. In particular, its secretion by other T lymphocytes recruited to the site during inflammation could reverse apoptosis, thereby enabling a response from T cells that were destined for termination. In the context of autoimmune inflammation, this would be an undesirable outcome. Such a possibility was examined by determining whether proliferative activity of T cells preexposed to MG and specific antigen and “rescued” with IL-2 could be restored by cross-linking with an anti-α/βTCR mAb (Fig. 4 B). According to the results, MBP T cells (as well as an OVA-reactive CD4 T cell line included to test the universality of the effect) responded poorly to this stimulus when the level of proliferation was compared with T cells derived from cultures containing LN APC. A third MBP-reactive T cell line gave a qualitatively identical response (not shown). Thus, IL-2–rescued T cells remained functionally anergic, at least on the basis of this assay. By analogy with anergic B and T cells in vivo, which exhibit shortened life spans relative to normal resting lymphocytes (2), the anergic state observed here may simply represent delayed death. Such a scenario in fact makes good sense, since persistence of populations of anergic T cells in tissues is of no obvious advantage to the host, and is consistent with previous reports of apoptotic T lymphocytes in CNS inflammation (37, 38).

GVHD-activated Microglia and CD45high CNS Macrophages Are B7.1/B7.2 Negative. The outcome of the encounter between T cells and antigen described here is determined entirely at the level of the APC. A possible explanation would be expression of costimulatory molecules by CD45high CNS macrophages but not MG. Occasional cells in the multiple sclerosis CNS (39) and in vitro–maintained human MG (40) reportedly are B7 positive. Here, however, a CTLA-4–Ig fusion protein that strongly labeled rat B cell blasts (Fig. 5 A, population 2) but not T cells (Fig. 5 A, population 1) or resting B cells (not shown) failed to detect B7.1 (CD80) or B7.2 (CD86) expression on freshly isolated MG or CD45high CNS macrophages from the normal (Fig. 5 B) and GVHD–affected CNS (Fig. 5 C). Note that when
Figure 5. Macrophage cell B7 expression. (A) Splenocytes cultured for 48 h on immobilized anti-rat k chain mAb to activate B cells (population 2). Population 2 was >90% B cell blasts, most macrophages remaining attached to the culture wells. (B and C) Freshly isolated MG (populations 3 and 4) and other CNS macrophages (populations 5 and 6). Solid histograms show B7.1 and B7.2 expression assessed using biotinylated human IgG. Dotted histograms represent the background fluorescence of designated populations stained with biotinylated human IgG.

stained only with a negative control reagent (dotted lines), a 5-10-fold increase in background fluorescence displayed by MG and other CNS macrophages (populations 3-6) relative to T cells and B cell blasts (populations 1 and 2) was observed. MG, like other macrophages are known to express Fc receptors and also are more autofluorescent than lymphocytes. These factors in combination may account for the background staining. Control experiments in which cultured splenocytes were exposed to the same enzyme treatment as that used to isolate cells from the CNS (17) showed that CTLA-4-Ig binding to B cell blasts was maintained.

Concluding Remarks. The processes responsible for elimination of potentially autoreactive T and B cells, both during development and after activation in lymphoid tissues, are increasingly well defined (33, 41). The role of lymphocyte Fas–FasL interactions is of particular importance. It has been speculated that mechanisms must also exist for termination of effector T cells in nonlymphoid tissues after antigen encounter (2, 3), although evidence for this and definition of the actual processes involved remains limited. In inflammation, the increased density of T cells and blood monocytes entering tissues, including the CNS through a disrupted blood brain barrier, may facilitate T cell regulation (33, 42) and potentially, at least, Fas–FasL interactions may participate. However, under conditions where T cells, including those with self specificity, traffic into and patrol normal tissues, it should not be assumed that apoptosis is mediated solely by interactions involving FasL (33) given that FasL expression appears quite restricted on resident tissue cells (43, 44). Nevertheless, FasL is now recognized to be present on a broader range of leukocytes than originally thought, e.g., mouse CD8+ DC (45), so the role of this molecule, as well as membrane TNF (46) known to be expressed strongly on macrophage lineage cells, requires investigation. Studies using MG derived from gld mice and also TNF-deficient mice recently produced by us (unpublished observations) are planned to extend the work done here in the rat model. It seems unlikely, however, that differential expression of these molecules alone will explain why non-MG CNS macrophages fully activate and maintain survival of CD4 T cells, while MG induce T cell apoptosis but at the same time support production by the T cell of the immunopathogenetically relevant cytokines TNF and IFN-γ. In this context, it is important to note that the inactivity of exogenous IL-2 in MG–T cell cultures to restore proliferation (Fig. 3 A), the requirement for antigenic stimulus by MG before substantial apoptosis is induced (Fig. 4 A), and the poor response to TCR cross-linking of IL-2–rescued T cells after MG interaction (Fig. 4 B), all argue strongly against the simplistic explanation that the consequences of MG–T cell interaction occur because MG lack the capacity to induce T cell IL-2 production.

In summary, the current findings support a role for fixed resident macrophages (in this case, CNS MG) as at least one way of controlling T cell responses in tissues, by inducing an immunological “swan song.” Such a mechanism could have a dual purpose. On the one hand, it may contribute to surveillance of tissues like the CNS, through controlled release by T cells of cytokines such as IFN-γ and TNF, but not IL-2 in view of its proliferative potential. On the other hand, it represents a novel way of terminating unwanted antself T cell responses during the normal course of immune surveillance, or within disease-affected inflamed tissues.

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