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Activation of Invariant NKT Cells in Early Phase of Experimental Autoimmune Encephalomyelitis Results in Differentiation of Ly6C\textsuperscript{hi} Inflammatory Monocyte to M2 Macrophages and Improved Outcome

Laura Denney, Wai Ling Kok, Suzanne L. Cole, Sharon Sanderson, Andrew J. McMichael, and Ling-Pei Ho

Neuropathology in multiple sclerosis is closely linked to presence of macrophages in the CNS. Both M1 (inflammatory) and M2 (alternatively activated, noninflammatory) macrophages are found in the inflamed CNS and thought to differentiate from infiltrating monocytes. It is unclear whether the balance of M1 and M2 macrophages can be altered and whether this affects disease outcome. We show in this article that Ly6C\textsuperscript{hi} inflammatory monocytes are the early and dominant infiltrating cells in the CNS during experimental autoimmune encephalomyelitis, a model for the acute phase of multiple sclerosis. Activation of invariant NKT (iNKT) cells reduced the frequency of Ly6C\textsuperscript{hi} monocytes and increased the proportion of M2 macrophages in the CNS with associated improvement in neurologic impairment. In contrast, iNKT-deficient mice showed higher numbers of Ly6C\textsuperscript{hi} monocytes, reduced M2, and much more severe disease. Adoptive transfer of M2-enriched cells to iNKT-deficient mice markedly improved neurologic impairment. In vitro and in vivo experiments showed that iNKT cells promote differentiation of monocytes to M2 macrophages in an IL-4 and CD1d-dependent process. These findings indicate that infiltrating Ly6C\textsuperscript{hi} inflammatory monocytes are early players in acute neuroinflammation and that their frequency and differentiation can be influenced by activation of iNKT cells with resultant improvement in disease outcome. The Journal of Immunology, 2012, 189: 551–557.

Macrophages and activated resident microglia play a major role in the inflammatory process of multiple sclerosis (MS) (1). Macrophages are thought to arise from the common monocyte, macrophage, and dendritic cell (DC) precursor and circulate as Ly6C\textsuperscript{hi} (GR1\textsuperscript{+}, inflammatory) monocytes (2). In contrast, Ly6C\textsuperscript{−}GR1\textsuperscript{−} monocytes do not infiltrate inflammatory sites but have a surveillance role and can “crawl” over long distances of the endothelium seeking signs of tissue damage or infection (3). Ly6C\textsuperscript{−} murine macrophages have been shown to migrate from the blood to tissues following infection, where they undergo activation and differentiation to different types of phagocytes—DCs, “TipDCs,” and macrophages (4). The consequence of this infiltration could be deleterious, for example, in the release of NO and TNF-α from TipDCs in severe influenza viral infection (5), or beneficial, as in a model of spinal cord injury where the recruitment of Ly6C\textsuperscript{−} macrophages aided tissue repair (6). A recent study has shown that infiltrating Ly6C\textsuperscript{−} monocytes have fundamentally different role from microglia (7). Although microgliosis could be an initial step in pathogenesis of experimental autoimmune encephalomyelitis (EAE), infiltrating monocytes appear more involved in the acute phase of EAE, which occurs as relapses in the relapsing-remitting form of the human disease (MS). Once monocytes infiltrate the CNS, they differentiate into macrophages, which can further be divided into M1 and M2 macrophages (8). The “classically activated” M1 macrophages produce high levels of oxidative metabolites (e.g., NO and superoxide) and proinflammatory cytokines that are essential for host defense and tumor cell killing but can also cause damage to healthy cells/tissue (9). Conversely, “alternatively activated” M2 phenotype, whose differentiation is driven by IL-4 or IL-13, promotes angiogenesis and matrix remodeling while suppressing destructive immunity. M2 macrophages are associated with tumor progression, some parasite and fungal infections, tissue repair, and debris removal (10). These can further be divided into subsets based on the stimuli that activate them. M2a macrophages are generated after exposure to IL-4 and/or IL-13, whereas M2b are driven by immune complexes in combination with IL-1β or LPS and M2c by IL-10, TGF-β, or glucocorticoid stimulation (11).

There is some evidence for increase in M1 macrophage populations in MS (8, 12, 13) but M1/M2 macrophage profile during disease genesis in MS and EAE has been poorly studied. It is unclear how this differentiation occurs, what factors influenced this process during disease and whether artificially manipulating the balance from one subset to the other affect outcome of acute neuroinflammation.

In this study, we question whether invariant NKT (iNKT) cells could influence the frequency of Ly6C\textsuperscript{−} inflammatory monocytes in the CNS during acute neuroinflammation and if they could change the differentiation path of these monocytes. iNKT cells are a small group of circulating leukocyte with immunoregulatory...
function. It acts rapidly upon ligation with its prototype glycolipid (α-galactosyl ceramide [α-GC]), but it is likely that there is a (as yet poorly characterized) self-ligand which keeps these cells under constant low-level activation (14). This low-level activity can be significantly enhanced during infection by IL-12 produced by DCs (15). We have recently shown that iNKT cells can interact with Ly6C<sup>+</sup> monocytes, resulting in reduction of Ly6C<sup>+</sup> monocytes numbers in lungs during severe influenza A virus infection (16). It is well established that iNKT cells can modulate disease severity in EAE (17–20, 22), and the frequency of these cells correlated positively with remission in MS (20). However, it is not clear how iNKT cells improved disease though this is IL-4 (and possibly IL-10) dependent because IL-4 and IL-10 knockout mice do not benefit from iNKT activation (17). In addition, an α-GC analog, OCH, which preferentially caused IL-4 production in vivo, was more effective than α-GC in preventing EAE (22).

In this study, using CD206 and Dectin-1 to characterize M2 macrophages, we show that iNKT activation results in both a reduction in the frequency of Ly6C<sup>+</sup> monocytes in the CNS and an M2 bias in these cells. Adaptoive transfer experiments, which controlled for monocyte numbers, suggest that it is the bias toward M2 that had the greater effect on outcome. This differentiation of Ly6C<sup>+</sup> monocytes to M2 macrophages is CD1d-dependent in vitro, and requires IL-4 but not IL-13. Thus we propose that during acute neuro-inflammation, activation of iNKT cells improves outcome by conditioning Ly6C<sup>+</sup> monocytes to differentiate to M2 macrophages in the CNS.

Materials and Methods

The EAE model was generated as described elsewhere (23). Myelin oligodendrocyte glycoprotein (MOG)<sub>35-55</sub> peptide MEGWYRSPSRVHLYRNGK was synthesized in-house by solid-phase 9-fluorenylmethoxycarbonyl chemistry (purity > 90%). C57BL/6 mice were obtained from breeding pairs originally purchased from the Jackson Laboratory. Jo1<sup>−/−</sup> mice were provided by Prof. M Taniguchi (RIKEN, Japan) and were backcrossed with C57BL/6 mice for 12 generations (24). All mice (8–10 wk, female) were maintained in a specific pathogen free environment at the biomedical facility (John Radcliffe Hospital, Oxford, U.K.). All animal work was carried out under license and in accordance with the Animals (Scientific Procedures) Act 1986.

All mice were injected s.c. in the flank with an emulsion of 200 μl MOG<sub>35-55</sub> peptide (1 mg/ml) and 200 μl CFA-4 mg/ml Mycobacterium tuberculosis H37Ra suspended in IFA (Sigma-Aldrich, Gillingham, Dorset, U.K.). Two hundred nanograms of pertussis toxin was injected into the tail vein on days 0 and 2 post-MOG administration. Where “MOG immunization” or “EAE mice” were mentioned in the text, this refers to injection of this adjuvant and pertussis toxin in addition to MOG peptides. Mice were examined daily for signs of EAE, and clinical symptoms were scored as described previously (25): briefly, 0.0, no disease; 0.5, partial tail paralysis; 1.0, complete tail paralysis; 1.5, complete tail paralysis and discrete hind weakness; 2.0, complete tail paralysis and strong hind weakness; 2.5, unilateral hind limb paralysis; 3.0, complete hind limb paralysis; 3.5, complete hind limb paralysis and forelimb weakness; and 4.0, Tetraplegia.

Two micrograms of α-GC (Alixes Biochemicals, Axxara, Nottingham, U.K.) was administered intraperitoneally at designated points; the buffer that suspends α-GC (0.5% Tween 20/150 mM NaCl in water) was used as control. Cells from the CNS were harvested from spinal cord, medulla oblongata, mesencephalon,pons, cerebellum, and brainstem. These were gently washed and resuspended in 5 ml 70% buffered Percoll (GE Healthcare, Chalfont, U.K.) layered with 4 ml 37% buffered Percoll and then 4 ml 30% buffered Percoll. After centrifugation, the interface containing lymphocytes was harvested, washed, and incubated in 3 ml RBC lysis buffer (RBClysis) (Qiagen, Crawley, U.K.).

Cytokine and chemokine protein concentrations were measured using a bead cytokine assay system, Bio-Plex 200 system (Bio-Rad, Hemel Hempstead, U.K.), according to the manufacturer’s instructions. All mAbs for flow cytometry were purchased from eBioscience (Hatfield, U.K.) apart from Dectin-1 (clone 2A11 from AB Serotec), Ly6G, and Ly6C (clone 1A8 and 1G7.G10, respectively; both from Miltenyi Biotec, Bisley, U.K.). Blocking CD1d mAb was the 3C11 clone from BD Biosciences (Oxford, U.K.).

Samples were analyzed on three-laser, nine-color CyAn ADP Flow Cytometers (DakoCytomation, Cambridgeshire, U.K.) using Summit 4.3 (DakoCytomation). Cells were sorted on MoFlo XDP Cell Sorter (Beckman Coulter, High Wycombe, U.K.).

Statistical analysis

Statistical tests used for each figure was stated in the figure legend. All data were tested for normality using the D’Agostino and Pearson omnibus normality test from the statistical package in GraphPad Prism 5, and parametric and nonparametric tests were used as appropriate. For analyses of percentages, the data were also log transformed to ensure that the “wall effects” at the extremes of the data do not affect the analytical outcomes.

Results

Ly6C<sup>+</sup> monocytes dominate infiltrating cells in the CNS, but its frequency can be modulated by a single, early point iNKT activation

Using a well-established protocol where MOG (23) was used to induce EAE in C57BL/6 mice, we first examined the composition of immune cell infiltrates in the CNS during disease genesis. We found that in the early phase (day 4, before manifestation of clinical signs), hematopoietic cells in the CNS can be divided into resident microglia (CD45<sup>−</sup>/<sup>−</sup>, and infiltrating cells (CD45<sup>+</sup> cells) (25) (Supplemental Fig. 1A). Nearly 60% of these CD45<sup>+</sup> infiltrating cells were Ly6G<sup>+</sup>Ly6C<sup>+</sup> inflammatory monocytes and Ly6G<sup>−</sup>Ly6C<sup>+</sup> neutrophils (see Supplemental Table 1 for markers differentiating the innate immune cells). Ly6C<sup>+</sup> inflammatory monocyte numbers were highest on day 4 in the CNS, whereas neutrophils became dominant only later in the disease (Fig. 1A, left panel). MOG-specific IL-17<sup>+</sup>IFN-γ<sup>+</sup> CD4 T cells and IFN-γ<sup>+</sup>TNF-α<sup>+</sup> CD8 T cells (Supplemental Fig. 1B) in the CNS were not detected until day 11, although CD3 T cells were present from day 4.

Because Ly6C<sup>+</sup> inflammatory monocytes were established in the CNS by day 4 after (MOG) immunization, we questioned whether iNKT cell activation early in the disease affected the frequency of these cells. We administered a single I.P. injection of α-GC on day 2 after MOG immunization (before manifestation of clinical signs) and found that this significantly reduced the number of Ly6C<sup>+</sup> inflammatory monocytes and severity of clinical disease (Fig. 1B, 1C). There was also a lower proportion of CD115 expressing Ly6C<sup>+</sup> inflammatory monocytes within this population of infiltrating cells (Fig. 1D). CD115 is expressed on the majority of cells designated as mononuclear phagocytes and is the receptor for M-CSF (CSF-1), a type III integral membrane protein tyrosine kinase (26). The ligand, CSF-1, controls the proliferation, differentiation, adaptation, and survival of cells of the mononuclear phagocyte system. Downregulation of this receptor is synonymous with activation of monocytes (27). Therefore, even though the numbers of inflammatory monocytes in CNS of α-GC–treated mice were reduced, in fact, a greater proportion of these cells were activated. To investigate whether iNKT cells have an effect on activation and infiltration of Ly6C<sup>+</sup> monocytes even without exogenous activation, we examined the frequency and CD115 expression on these monocytes in CNS of iNKT-deficient (Jo1<sup>−/−</sup>) mice at the same point (4 d) after MOG immunization. We found an increase in Ly6C<sup>+</sup> monocytes, with even higher CD115 expression in iNKT-deficient mice with EAE (Fig. 1D). The latter suggests that under steady-state conditions, iNKT cells continually engaged with Ly6C<sup>+</sup> monocytes and imposed a low-level check on their function and infiltration.
different days. The analyzed using two-way ANOVA with repeated measures to accommodate different treatments and days after immunization, and repeated measures on Ly6Chi monocytes found in CNS on day 4 after MOG immunization. There was significant difference (without administration of \( \alpha \)-GC–treated wells showed significantly reduced \( p \), \( \alpha \)-GC–treated spleen cultures (Supplemental Fig. 1C, 1D). Together with downregulation of CD115 expression (Fig. 2F). As in the in vitro studies, presence of iNKT cells alone had an effect on the differentiation of inflammatory monocytes because iNKT-deficient mice had even less M2 macrophages (Fig. 2F).

pairment with a large proportion of mice progressing to clinical score 4 (tetraplegia, because ascending paralysis), and requiring humane sacrifice (Fig. 1E, 1F).

**iNKT cell activation promote differentiation of Ly6C\(^{hi}\) inflammatory monocytes to M2 macrophages in vitro and in vivo**

Because previous studies have shown that iNKT-associated improvement in EAE is IL-4 dependent (17, 22), and we have observed that in vivo activation of iNKT cells is associated with activation of Ly6C\(^{hi}\) inflammatory monocytes, we hypothesize that iNKT cells might deviate the differentiation of monocytes to M2 macrophages. To investigate this, and to confirm that CD115 changes observed on inflammatory monocytes in the CNS was not due to efflux of these cells from this site, we examined the effect of iNKT cells activation on inflammatory monocytes in a “closed system” in vitro. For this, splenocytes (shown to contain Ly6C\(^{hi}\) inflammatory monocytes and iNKT cells) from EAE mice (day 4) were halved, placed in two wells, and incubated with \( \alpha \)-GC or control (vehicle used to suspend \( \alpha \)-GC), for 7 h (Fig. 2A), and stained for flow cytometry examination of the cells. Inflammatory monocytes in the \( \alpha \)-GC–treated wells showed significantly reduced CD115 expression (Fig. 2B, 2D) confirming that iNKT cell activation results in activation of Ly6C\(^{hi}\) inflammatory monocytes. There was no change in numbers of Hoechst\(^{+}\) cells or Hoechst\(^{+}\) inflammatory monocytes in the wells at the seventh hour, indicating no excess death in \( \alpha \)-GC–treated spleen cultures (Supplemental Fig. 1C, 1D). At the same time, we found that Dectin-1\(^{hi}\) and CD206\(^{hi}\) (markers for M2 or alternatively activated macrophages (27–31); see also Supplemental Fig. 1E for CD206 expression) monocyte-macrophages (F4/80\(^{+}\)Ly6C\(^{hi}\)CD11b\(^{+}\) cells) were markedly increased after \( \alpha \)-GC incubation (Fig. 2C, 2E). Together with downregulation of CD115 (Fig. 2D), this indicates that iNKT cell activation is associated with activation of Ly6C\(^{hi}\) inflammatory monocytes and a change in M1/M2 differentiation pathway toward an M2 bias.

We next investigated whether this was also the case in vivo by examining M1/M2 bias in the CNS of mice with EAE, 4 d after immunization, comparing wild-type mice given \( \alpha \)-GC to wild-type control and iNKT-deficient (Jax18\(^{−/−}\)) mice. Absolute numbers of M2 macrophages were increased in CNS of \( \alpha \)-GC treated mice (Fig. 2F). As in the in vitro studies, presence of iNKT cells alone had an effect on the differentiation of inflammatory monocytes because iNKT-deficient mice had even less M2 macrophages (Fig. 2F).
Ly6Chi monocytes in CNS of mice with EAE – WT given To investigate whether a EAE reduced neurologic impairment in iNKT-deficient mice with Administration of a 3F). phages to levels observed in mice that did not receive a with CNS when IL-4–blocking Abs were concomitantly administered (Fig. 3C). Coculture with recombinant IL-4 (but not IL-13), significantly increased frequency of Dectin-1hi cells (Fig. 3D). Conversely, addition of IL-4 blocking Abs (but not IL-13 blocking Abs) to the short-term spleen culture system as used in Fig. 2A after incubation with α-GC or control, significantly increased frequency of Dectin-1hi cells (Fig. 3D). These findings indicate that IL-4 is required for differentiation of monocytes to M2 macrophages in this splenic culture and that this is CD11d-dependent, which suggests that the source of IL-4 is iNKT cells. To question whether IL-4 is involved in the observed increase in M2 macrophages in CNS of EAE mice given α-GC, we examined the numbers of M2 macrophages in the CNS when IL-4-blocking Abs were concomitantly administered with α-GC. This resulted in reduced frequency in M2 macrophages to levels observed in mice that did not receive α-GC (Fig. 3F).

Administration of α-GC-conditioned monocytes markedly reduced neurologic impairment in iNKT-deficient mice with EAE
To investigate whether α-GC-conditioned, Dectin-1hi (M2)-enriched monocytes can rescue the neurologic impairment observed in Jax1818−/− EAE mice, we isolated monocytes from a short-term spleen culture system as used in Fig. 2A after incubation with α-GC or control and adoptively transferred this to iNKT-deficient mice with EAE. Monocytes were derived by first depleting splenocytes of Ly6G+ cells and then positively isolating CD11b+ cells using Miltenyi microbeads. These cells were shown post isolation to be Ly6Chi CD115lo, and enriched for Dectin-1hi monocytes (Fig. 4B, 4C). Survival and mean clinical score were determined as in Fig. 1E and 1F.

Discussion
The pathogenesis of MS has been extensively studied. It is thought that initiation of MS is triggered by myelin-specific Th1 or Th17 cells, which in turn generate the expansion of resident microglia and infiltration of monocytes (32–34). The latter groups of cells appear crucial in determining the severity of the acute inflammatory phase of the disease. Contribution of monocytes to the acute inflammatory phase and progression of disease has recently been strengthened by a group using parabiotic animals to show that compromise of the blood–brain barrier and subsequent invasion of the CNS by inflammatory monocytes are the key events in promoting disease progression in EAE (7). The EAE model is not an exact model of the human disease, particularly in terms of the natural clinical progression. However, it is a well-regarded model of the acute neuroinflammatory phase of MS, which occurs at the point of disease genesis and later during acute relapses in the relapsing and remitting phase. Our study suggests that the expansion of myelin-specific T cells is a late phenomenon; rather, Ly6Chi inflammatory monocytes dominate the acute phase of EAE and this is reduced when iNKT cells are activated. iNKT cells also promote a M2 macrophage bias in the differentiation of monocytes, and adoptive transfer of M2-enriched cells to iNKT-deficient mice reduced disease severity and mortality de-
spite increased inflammatory monocyte levels in the CNS of these mice. This suggests that M2 biasing of infiltrating monocytes is more important than reduction in inflammatory monocytes in providing protection after iNKT cell activation.

M2 or alternatively activated macrophages are a relatively new concept, which has gained increasing credibility in the last decade both for mice and human cells. It encompasses an alternative pathway of macrophage activation induced by the Th2 cytokines and is distinct from IFN-γ-mediated classical activation (28, 10). These monocytes can be defined by markers like Fizz-1, Ym-1 and 2, Arg1, CD206 (macrophage mannose receptor or MRC-1), and Dectin-1 (10, 28–30). In our study, we used Dectin-1 and CD206 because these can be detected by flow cytometry, allowing monocyte differentiation to be tracked alongside examination of changes in other cells.

M2 macrophages are implicated in a range of physiologic and pathological processes, including homeostasis, inflammation, repair, metabolic functions, and malignancy, and its role is mainly anti-inflammatory (36). Although their differentiation is thought to be dependent on IL-4 and IL-13, our data suggest that IL-13 is not crucial (Fig. 3C). This fits with the concept that monocytes are more sensitive to IL-4 than IL-13 (35). The high levels of IL-4 after activation of iNKT cells in our short-term spleen culture (Fig. 3A) could have abrogated the need for IL-13.

Our findings suggest that the deviation in differentiation is likely to have occurred in the periphery because no iNKT cells were detected in the CNS during the early part of disease (or indeed at any other point) (Supplemental Fig. 2A), both with and without α-GC administration. This means that the Ly6Chi cells infiltrate the CNS after commencement of differentiation in a peripheral site, possibly a secondary lymphoid organ, and rely on the breakdown on blood–brain barrier to infiltrate the brain. The study was not able to specify the precise site of interaction between iNKT cells and Ly6Chi monocytes. Our in vitro studies suggest that the spleen is a likely site for this interaction, although this can equally occur in the draining lymph nodes because both these sites showed an increase in iNKT cells on day 4 after MOG immunization (Supplemental Fig. 2A), and presence of Ly6Chi monocytes at the same time (Supplemental Fig. 2B). However, the numbers of iNKT cells are very small in the draining lymph nodes compared with the spleen. More work is required in this area to pinpoint this site. Delineating this could be important because it could mean a clinically viable pathway for new therapeutic strategies in the disease. As the monocytes are accessible in the blood, they can be modulated peripherally and monitoring of the effect of therapy can be done without access to the CNS. The adoptive transfer experiments showed marked improvement in disease score in the first phase of clinical disease (from days 10 to 15) when both...
weight loss and clinical score were significantly reduced after transfer. This appears to require only one point of influence. This bodes well for potential translation to human therapy because this implies that a single point of manipulation may be sufficient to change the course of the disease. Moreover, because monocytes are replenished from the bone marrow, fresh monocytes can be targeted for treatment depending on clinical need. One caveat, however, is that the point of manipulation in our study occurs before the manifestation of clinical signs so that neurologic impairment cannot be the cue for intervention. Rather, the behavior of circulating monocytes or iNKT may have to be the signal for intervention. For example, circulating iNKT cells has been shown to be lowest just before a relapse in MS (21). In this scenario, administration of IL-4 without the need for activation of iNKT cells (as shown in Fig. 3D) could overcome the lack of these cells. Another point to note is that the protection is transient, limited to the early phase of clinical disease. This may be due to the lifespan of these inflammatory monocytes and could be a positive thing in a clinical scenario, because it is much safer to induce a transient change in immune function rather than a long-term one.

The ability of nonactivated iNKT cells to provide a baseline “check” on infiltration of inflammatory monocytes to the CNS during EAE is interesting and suggested by changes observed in iNKT-deficient mice compared with untreated wild-type mice with the disease. This observation in iNKT cells and the subsequent rescue of neurologic impairment by transfer of α-GC–conditioned monocytes implies that iNKT cells have a role in moderating the inflammatory phase of EAE and do this by regulating the function of monocytes. Moreover, this seems a significant contribution because the consequence of iNKT loss is severe (Fig. 1E, 1F). However, our study does not prove that iNKT cells alone modified the differentiation pathway, nor that it acts directly on inflammatory monocytes; rather, that iNKT cells are at least partially involved (because CD1d blockade in vitro reduced M2 bias) and that activation of iNKT cells results in downstream change in the monocyte differentiation pathway. Indeed, iNKT cells have been shown to influence other groups of immune cells that could moderate inflammation [e.g., tolerogenic DCs (36)].

In conclusion, we show that a single point of activation of iNKT cells during acute neuroinflammation is sufficient to facilitate the course of differentiation of Ly6C^{hi} inflammatory monocytes, biasing it toward M2 macrophages with consequent reduction in neurologic deficit. This process relies on IL-4 and offers a potential extension to the IL-4–dependent mechanism for iNKT cell mediated protection observed in EAE mice. It provides a platform for more research to explore studies leading to potential use of a single administration of α-GC or IL-4 to patients with relapsing-remitting MS to moderate the severity of the relapse. Finally, it shows another consequence of the interaction between iNKT cells and monocytes and highlights the role of this interaction in disease.

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Disclosures

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