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*J Immunol* 2012; 189:551-557; Prepublished online 8 June 2012; doi: 10.4049/jimmunol.1103608

http://www.jimmunol.org/content/189/2/551

**Supplementary Material**  
http://www.jimmunol.org/content/suppl/2012/06/08/jimmunol.1103608.DC1

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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 macrocytes 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.
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>hi</sup> monocytes, resulting in reduction of Ly6C<sup>hi</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 with EAE do not benefit from iNKT activation (17). In addition, an α-GC analog, OCH, which preferentially induced 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 vivo, was more effective than α-GC in preventing EAE (22).

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**Materials and Methods**

The EAE model was generated as described elsewhere (23). Myelin oligodendrocyte glycoprotein (MOG<sub>35-55</sub>) peptide MEVGYSPLPSRSVH-LYRNGK was synthesized in-house by solid-phase 9-fluorenylmethoxy-carbonyl chemistry (purity > 90%). C57BL/6 mice were obtained from breeding pairs originally purchased from the Jackson Laboratory. J<sub>18</sub> (<sup>129</sup>Sn) 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 forelimb weakness; and 2.0, complete tail paralysis and hind limb weakness. Percentage expression on these monocytes in CNS of iNKT-deficient (J<sub>18</sub> (<sup>129</sup>Sn)) mice was found to be significantly reduced, in fact, a greater proportion of these cells were activated. To investigate whether iNKT cells have an effect on 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 with EAE do not benefit from iNKT activation (17). In addition, an α-GC analog, OCH, which preferentially induced IL-4 production in vivo, was more effective than α-GC in preventing EAE (22).

Results

**Ly6C<sup>hi</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>CD11b<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>hi</sup> inflammatory monocytes and Ly6G<sup>−</sup>Ly6C<sup>−</sup> neutrophils (see Supplemental Table I for markers differentiating the innate immune cells). Ly6C<sup>hi</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>hi</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>hi</sup> inflammatory monocytes and severity of clinical disease (Fig. 1B, 1C). There was also a lower proportion of CD115 expressing Ly6C<sup>hi</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>hi</sup> monocytes even without exogenous activation, we examined the frequency and CD115 expression on these monocytes in CNS of iNKT-deficient (J<sub>18</sub> (<sup>129</sup>Sn)) mice at the same point (4 d) after MOG immunization. We found an increase in Ly6C<sup>hi</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>hi</sup> monocytes and imposed a low-level check on their function and infiltration. Lack of iNKT cells during EAE disease resulted in a marked increase in neurologic im-
Influence of iNKT cells on inflammatory monocytes and disease outcome in EAE. (A) Left panel, Frequency of inflammatory monocyte, neutrophil, and macrophages (defined in Supplemental Table I) in CNS on days 4, 11, and 15 after MOG immunization. The p values refer to differences between the three cell populations on designated days, analyzed by one-way ANOVA. Posttest analysis with Tukey test showed inflammatory monocytes to be significantly (*p < 0.05) raised compared with the rest on D4, while neutrophils were significantly raised on D11 and D15. A similar analysis was performed for absolute numbers of cells with the same results. Right panel, Representative FACS plot of CNS on day 4 after MOG immunization in wild-type (WT) mouse showing that CD45hi CD3neg (infiltrating cells other than T lymphocytes) cells making up 58% of cells derived from the CNS (top panel; note CD45mid cells, in box of broken line, are likely to represent microglia and was excluded from analysis). Bottom panel shows the percentage of Ly6G and Ly6C staining of these CD45hi CD3neg cells. (B) Inflammatory monocyte numbers in the CNS of mice on day 4 after administration of α-GC on day 2 in WT mice with EAE. The p value was determined by Student t test. (C) Clinical EAE scores (mean ± SEM) over time in WT mice with and without administration of α-GC on day 2. Clinical score of 4 was never reached, and there were no deaths. Difference between the two groups was analyzed using two-way ANOVA with repeated measures to accommodate different treatments and days after immunization, and repeated measures on different days. The p value refers to difference between the two groups. n = 18 mice/group, two independent experiments. (D) CD115 expression on Ly6C+ monocytes found in CNS on day 4 after MOG immunization. There was significant difference (p < 0.001) between the groups, determined by one-way ANOVA; posttest analysis with Tukey test showed significant differences (p < 0.05) between all pairs of groups, denoted by asterisks. (E) Survival curve of WT versus Jα18–/– mice with EAE. Death was actively “managed” by humanely sacrificing mice that reached clinical score 4 (tetraplegia) and were clearly dying from ascending flaccid paralysis (p value was analyzed with log-rank Mantel–Cox test). n = 20 mice/group, two independent experiments. (F) Clinical EAE scores (mean ± SEM) over time in WT and Jα18–/– mice that survived the disease. Data were analyzed using two-way ANOVA (mice that died/culled were scored as 4 in the statistical analysis to maintain matched number in both groups; but not presented in the graph to prevent biased appearance).

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+ 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+ 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+ inflammatory monocytes and iNKT cells) from EAE mice (day 4) were halved, placed in two wells, and incubated with α-GC or control (vehicle used to suspend α-GC), for 7 h (Fig. 2A), and stained for flow cytometry examination of the cells. Inflammatory monocytes in the α-GC–treated wells showed significantly reduced CD115 expression (Fig. 2B, 2D) confirming that iNKT cell activation results in activation of Ly6C+ 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 α-GC–treated spleen cultures (Supplemental Fig. 1C, 1D). At the same time, we found that Dectin-1hi and CD206hi (markers for M2 or alternatively activated macrophages (27–31); see also Supplemental Fig. 1E for CD206 expression) monocyte-macrophages (F4/80+Ly6C+CD11b+ cells) were markedly increased after α-GC incubation (Fig. 2C, 2E). Together with downregulation of CD115 (Fig. 2D), this indicates that iNKT cell activation is associated with activation of Ly6C+ 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 α-GC to wild-type control and iNKT-deficient (Jα18–/–) mice. Absolute numbers of M2 macrophages were increased in CNS of α-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 EAE reduced neurologic impairment in iNKT-deficient mice with a Administration of 3F). Phages to levels observed in mice that did not receive a CNS when IL-4–blocking Abs were concomitantly administered (performed on the fourth day after MOG immunization. Difference between groups was determined using one-way ANOVA (numbers did not change in the two wells over 7 h (Supplemental Fig. 1A, 1B). Frequency of CD115+ monocytes (Fig. 3E) at the seventh hour after incubation with α-GC or control. The p values in (D) and (E) were analyzed with Student t test. (F) Dectin-1hi expressing Ly6C+ monocytes in CNS of mice with EAE – WT given α-GC i.p. on day 2 and Jq18+/−/− (iNKT knockout [KO]) compared with WT. Sampling was performed on the fourth day after MOG immunization. Difference between groups was determined using one-way ANOVA (p < 0.001). Posttest analysis using Tukey’s test showed significant difference between all groups against each other; *p < 0.005.

**FIGURE 2.** Activation of iNKT cells increased differentiation of inflammatory monocytes to M2 macrophages in vitro and in vivo. (A) Experimental path for (B)–(E) and Fig. 3. Spleens were harvested from wild-type (WT) mice 4 d after immunization with MOG and divided in equal cell numbers (0.5 × 106 cells) into 2 wells and incubated with 0.2 μg/ml α-GC or control for 7 h. (B) Representative FACS plots for CD115 (B) and Dectin-1 (C) expression on Ly6C+ monocytes at the seventh hour from the two wells. The percentage refers to positive cells as proportion of Ly6C+ monocyte. Live (Hoescht−) cell numbers did not change in the two wells over 7 h (Supplemental Fig. 1A, 1B). Frequency of CD115+ monocytes (D) and Dectin-1hi expressing monocytes (E) at the seventh hour after incubation with α-GC or control. The p values in (D) and (E) were analyzed with Student t test. (F) Dectin-1hi expressing Ly6C+ monocytes in CNS of mice with EAE – WT given α-GC i.p. on day 2 and Jq18+/−/− (iNKT knockout [KO]) compared with WT. Sampling was performed on the fourth day after MOG immunization. Difference between groups was determined using one-way ANOVA (p < 0.001). Posttest analysis using Tukey’s test showed significant difference between all groups against each other; *p < 0.005.

**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, Ly6C+ 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 Ly6C<sup>hi</sup> 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 Ly6C<sup>hi</sup> 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 Ly6C<sup>hi</sup> 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

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**FIGURE 3.** Contribution of IL-4 to differentiation of monocytes to M2 macrophage after iNKT activation during EAE. (A) Cytokine levels in supernatant at the seventh hour after incubation with α-GC or control (“+0”), from experiment described in Fig. 2A. The p values were derived using Mann–Whitney U test. (B and C) IL-4 concentration in supernatant and frequency of Dectin-1<sup>hi</sup> expressing cells in wells with control (“+0”) versus with α-GC after 7 h culture with increasing doses of CD1d blocking Abs (“αCD1d”). The p values depict significant overall difference between groups using one-way ANOVA with repeated measures. Untreated cells form the short-term spleen cultures are shown for comparison in (C) but not included in the statistical analysis. (D–F) Frequency of Dectin-1<sup>hi</sup> expressing cell frequency at the seventh hour of the experiment, with and without addition of 0.2 mcg/ml α-GC and 10 ng/ml recombinant IL-4 or IL-13 or both; or IL-13 and IL-4 blocking Abs. In (E), blocking Abs for IL-4 and IL-13 (2 μg/ml each) were added from the start of the experiments. (F) Numbers of Dectin-1<sup>hi</sup> expressing cell in CNS of mice with EAE after administration of 50 μg of IL-4 blocking Ab, given i.v. at the same time as α-GC. The p values for (D–F) refer to overall difference between groups determined with one-way ANOVA, individual horizontal bars identify pairs of groups that were significantly different using posttest analysis (Tukey’s). (D and E) *p < 0.05, refers to pairwise comparisons that were significantly different.
between Jα18−/− mice. In (B), depiction of clinical scoring was curtailed at the 13th day because mice that scored 4 were sacrificed, and almost all had been sacrificed in the untreated or mock-treated groups by Day 14. The p value in (B) was calculated using two-way ANOVA repeated measures as in Fig. 1C and 1F, except that Bonferroni was applied for multiple comparisons between the three groups to detect difference in score. The p value in (B) refers to difference in clinical score between Jα18−/− mice immunized with MOG (“Jα18−/− EAE”), which received control-conditioned monocytes versus Jα18−/− mice immunized with MOG, which received α-GC–conditioned monocytes, on days 12 and 13. **p value in (C) (log-rank Mantel–Cox test) refers to difference between Jα18−/− EAE + control-conditioned monocytes versus Jα18−/− EAE + α-GC–conditioned monocytes. No difference was observed between Jα18−/− EAE group and those that received control instead of α-GC–conditioned monocytes. n = 7 in each group from two independent experiments.

In conclusion, we show that a single point of activation of iNKT cells during acute neuroinflammation is sufficient to deviate the course of differentiation of Ly6Chi 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.

Acknowledgments
We thank Prof. Vincenzo Cerundolo and Dr. Sandra Vergo for general discussions and Drs. Manuel Berdoy and Eleni Giannoulatou for in-depth statistical deliberations.
**Supplemental Figure 1.** (a) CD45 and CD11b expressing cells in CNS of EAE mice on day 4 after MOG immunization. Note that fluorescent conjugates are different from that in Figure 1a and provides a clearer picture of CD45 hi and lo separation. (i) refers to microglia and (ii) to infiltrating hematopoietic cells. (b) IL17+IFNγ+ CD4 cells and IFNγ+TNFα+CD8 cells in the CNS on Days 4, 11 and 15 after MOG immunization. In these experiments, CNS was harvested, mononuclear cells extracted and incubated with MOG (or no) peptide for 72 hours. Results for Day 15 no peptide control ("no MOG") is shown. In some mice, no clinical disease develops (less than 2% of mice) ("no clinical disease") in spite of immunization with MOG. In these mice, we never detected IL17+IFNγ+ CD4 cells or IFNγ+TNFα+CD8 cells in the CNS at any point. A representative plot on Day 15 is shown for these mice. (c) Amount of dead cells (Hoescht+) as proportion of total cells for experiment described in Figure 2(a) (at 7th hour). (d) Amount of dead cells (Hoescht+) as proportion of monocyte and macrophages in the same experiment (at 7th hour). (e) Graph showing CD206 expression (compared to isotype control) for Dectin-1 hi and Dectin-1 lo cells in the short term spleen culture as used in Figure 2A.

**Supplemental Figure 2.** (a) iNKT cell frequency in CNS, spleen and draining lymph nodes during EAE disease course. Upper panel of graphs shows frequency of iNKT cells as a percentage of CD3+ cell population in CNS (none observed), spleen and draining lymph nodes. (b) Frequency of CD3+ cell population as a percentage of mononuclear cells in the spleen – there was no difference between groups for CD3 measurements in spleen (p=0.078 with one-way ANOVA), hence the use of % of CD3 as denominator for iNKT cells). n=3 in each experiment; 3 experiments. DLN – draining lymph node; IC – isotype control. (c) Number of inflammatory monocytes in
CNS, spleen and draining lymph nodes showing presence of monocytes at all time points tested. Reduction in inflammatory monocyte numbers in early time point in periphery likely represent migration to inflamed CNS. The only significant difference between αGC and untreated mice was found in inflammatory monocytes numbers in CNS at Day 4.
Supplementary Figure 1
Supplemental Figure 2
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Supplementary Table 1