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Inhibition of Granulocyte-Macrophage Colony-Stimulating Factor Signaling and Microglial Proliferation by Anti-CD45RO: Role of Hck Tyrosine Kinase and Phosphatidylinositol 3-Kinase/Akt

Hyeon-Sook Suh, Mee-Ohk Kim, and Sunhee C. Lee

Increasing evidence suggests that CD45, a transmembrane protein tyrosine phosphatase, is an important modulator of macrophage activation. Microglia, resident brain macrophages, express CD45 and proliferate under pathologic conditions. In this study, we examined the role of CD45 in modulating GM-CSF-induced proliferation and signal transduction in primary human microglial cultures. Soluble, but not immobilized anti-CD45RO induced tyrosine phosphatase activity and inhibited GM-CSF-induced microglial proliferation. Microglial proliferation was also inhibited by PP2 (Src inhibitor), LY294002 (PI3K inhibitor), and U0126 (MEK inhibitor). GM-CSF induced phosphorylation of Jak2, Stat5, Hck (the myeloid-restricted Src kinase), Akt, Stat3, and Erk MAPks in microglia. Of these, anti-CD45RO inhibited phosphorylation of Hck and Akt, and PP2 inhibited phosphorylation of Hck and Akt. In a macrophage cell line stably overexpressing wild-type or kinase-inactive Hck, GM-CSF increased proliferation of the control (empty vector) and wild-type but not kinase-inactive cells, and this was inhibited by anti-CD45RO. Together, these results demonstrate that, in macrophages, Hck tyrosine kinase is activated by GM-CSF, and that Hck plays a pivotal role in cell proliferation and survival by activating the PI3K/Akt pathway. Ab-mediated activation of macrophage and microglial CD45 tyrosine phosphatase may have therapeutic implications for CNS inflammatory diseases. The Journal of Immunology, 2005, 174: 2712–2719.

Microglia are resident brain macrophages essential for the maintenance of normal brain physiology as well as a response to injury (1, 2). Microglia are activated in several CNS disorders of varying etiologies such as degenerative, inflammatory, infectious, and ischemic disorders. Mounting evidence indicates that activated microglia contribute to neuronal injury by producing inflammatory and neurotoxic mediators (1, 3, 4). Suppression of microglial activation may thus lead to reduction of neuronal damage. The attributes of activated microglia in vivo include ameboid transformation, increased (or de novo) expression of activation Ags such as class II MHC, scavenger receptors, CD11b/c and CD45 (5, 6), and proliferation. Although microglia in mature CNS are considered terminally differentiated, they can reenter the cell cycle following injury. In the rat facial axotomy model, microglia proliferate in the regenerating facial nucleus coinciding with the increase in CSF receptor expression (7, 8). Microglia are the main dividing CNS cell type in experimental allergic encephalomyelitis (9), cortical stab wound model (10), and experimental Wallerian degeneration (11). Microglia proliferate in response to growth factors such as GM-CSF and M-CSF that are produced by activated astrocytes, macrophages and microglia, and inflammatory cells (12–15).

Although GM-CSF is an important growth factor for microglia and macrophages, little is known about the mechanism by which GM-CSF induces proliferation of primary human macrophages. The GM-CSF receptor consists of a low-affinity α subunit (CD116) specific to GM-CSF and a high-affinity βc subunit (CD131) that is shared with IL-3 and IL-5 (16–19). βc lacks intrinsic kinase activity but ligand binding induces tyrosine phosphorylation of βc as well as a number of cytoplasmic proteins including various kinases and adaptor proteins. This results in the activation of several signaling pathways including the Jak/Stat, Ras/Erk MAPK, and the PI3K/Akt pathway (for review, see Refs. 16, 20, and 21). In addition, serine phosphorylation (S585) of the common β chain (βc chain)4 has been shown to mediate cell survival through activation of NF-κB and induction of the antiapoptotic protein bcl-2 (22, 23). Because many of these studies are performed in murine cells or cell lines with overexpression of receptor proteins or signaling molecules, we examined the GM-CSF signal transduction pathway involved in proliferation of primary human microglial cells.

CD45 is a transmembrane protein tyrosine phosphatase (PTPase) expressed in all hemopoietic cells including lymphocytes, monocytes, and granulocytes (24–26). Several isofoms of CD45 exist as a result of the alternative splicing of exons that encode the extracellular domain. All CD45 isoforms possess identical transmembrane and a cytoplasmic tail with two tandem PT-Pase homology domains (D1 and D2). CD45 enhances T cell and

4 Abbreviations used in this paper: βc chain, common β chain; PTPase, protein tyrosine phosphatase; WT, wild type; KE, kinase inactive; p, phosphorylated; T, total; Grb2, growth factor receptor-bound protein 2.

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purity, consisting of 0.1 ml of medium. Two to 4 h later, cultures were washed twice to remove per ml in DMEM (Cellgro: supplemented with 4.5 g/L glucose, 4 mM microglial cells were collected by aspiration of the culture medium. Mono-
ler targets of CD45: they reported that CD45 suppresses JAK activity, thereby negatively regulating cytokine and IFN signaling.

Although much study has been done on the role of CD45 in lymphocyte physiology, relatively little is known about the expres-
expression and function of CD45 in cells of the monocyte lineage. In the CNS, expression of CD45 by intrinsic brain microglia has been debated. Whereas several studies of macaques and humans ex-
cluded CD45 expression by microglia (40, 41), others, including our own, revealed that CD45 is expressed by microglia (5, 6). The different results may have been due to the low sensitivity of the techniques adopted in the former studies, as well as possible species differences. Although there is no consensus regarding expres-
sion, the functional significance of microglial CD45 has been clearly demonstrated in several instances. Using microglia derived from CD45-null mice, Tan et al. (42, 43) have shown that CD45 suppresses cytokine and NO production triggered by B-amyloid or CD40L. Furthermore, we find that anti-CD45 Ab suppresses HIV-1 production by human microglia (M. O. Kim and S. C. Lee, unpublished observations). Together, these results demonstrate that microglial cells express functional CD45 and that microglial CD45 activity can be manipulated to down-regulate brain inflammation. The present study is a further attempt to define the role of CD45 in the regulation of human microglial function. In this study, we report that Ab-mediated activation of microglial CD45 PTPase inhibits GM-CSF-induced cell proliferation. We further show that anti-CD45 Ab inhibited the myeloid-restricted Src kinase, Hck, and the downstream PI3K/Akt pathway.

Materials and Methods

Human fetal microglial culture

Human CNS cell cultures were prepared from human fetal abortuses as described (14, 44). All tissue collection was approved by the Albert Einstein College of Medicine Institutional Review Board. Primary mixed CNS cultures were prepared by enzymatic and mechanical dissociation of the cerebral tissue followed by filtration through nylon meshes of 230- and 130-μm pore sizes. Single-cell suspension was plated at 1–10 × 10^6 cells per ml in DMEM (Cellgro: supplemented with 4.5 g/L glucose, 4 mM t-glutamine, and 25 mM HEPES) supplemented 5% FCS (FCS: Gemini Bio-products), penicillin (100 U/ml), streptomycin (100 μg/ml), and fun-
gizone (0.25 μg/ml) (Invitrogen Life Technologies) for 2 wk, and then microglial cells were collected by aspiration of the culture medium. Mono-

CD45 Abs

Anti-CD45RO (clone UCHL-1) was obtained from either DakoCytomation or BD Pharamingen, mAbs against human CD45RA (clone HI100) and CD45RB (MT4) and all isotype-matched normal murine IgG were ob-
tained from BD Pharamingen.

Kinase inhibitors

Pharmacological inhibitors (45, 46) of Src kinase (PP2: 4-amino-5-(4-chlo-
rophophenyl)-7-(t-buty)pyrazole,3,4-dipyrimidine), PI3K (LY294002), or MEK1 (upstream of Erk MAPK: U0126) were obtained from Calbiochem. Stock solutions were made in DMSO and further dilutions were made in DMEM-containing serum. Vehicle controls were used to determine the specificity of the drug.

Cell treatment

Microglial cells in complete medium (DMEM plus 5% FCS) were treated with anti-CD45RO (or control Ig) at 5 μg/ml simultaneously or 30 min before addition of recombinant human GM-CSF (PeproTech) at 10 ng/ml (14). For kinase inhibitor experiments, cultures were simultaneously treated with the drugs and GM-CSF for 72 or 96 h before Ki67 immuno-
cytochemistry. For Western blot analysis, cells were treated with kinase inhibitors for 1 h or with Abs for 30 min, and then with GM-CSF for the indicated time periods.

Ki67 and CD68 immunocytochemistry

Microglial proliferation was determined by Ki67 immunocytochemistry at 72 or 96 h after GM-CSF treatment (14). Ki67 is a nuclear Ag expressed in all phases of cell cycle except G0, (47, 48). Cells were fixed with ice-cold methanol for 20 min, and then incubated with rabbit anti-human Ki67 (US Biological) at 1:100 at 4°C overnight, and then with biotinylated anti-rabbit IgG at 1:300 or 1:350 at room temperature for 1 h, followed by HRP-
labeled streptavidin. Color was developed with diaminobenzidine (brown nuclei: Ki67+). Double immunocytochemistry for Ki67, and CD68, a marker for macrophages and microglia, was performed. Cells stained first for Ki67 using DAB, and then sequentially for CD68 using anti-CD68 (KP1: DakoCytomation mouse IgG1) at 1:600 at 4°C overnight using the ABC (avidin/biotin complex) methods as described above. Color was de-
veloped using NBT/4-chloro-3-indolyl phosphate (purple cyto-
plasm: CD68-).

Cell counting

Ki67+ cells (nuclei) were counted from each well in eight random ×200 magnification fields using a Zeiss Axiosvert inverted microscope. Data are expressed as the average percentage of Ki67+ cells in each well. Ki67+ cells in control microglial cultures (without GM-CSF) were <1%; follow-
ging GM-CSF stimulation, ~10% cells were positive for Ki67 at 72 or 96 h. Each data point represents the mean ± SD from three to four wells from a single experimental group from the same brain case.

Western blot analysis

Abs against cell signaling molecules (phosphorylated (p) and total (T) Akt, Stat3, Stat5, ERK, Src, and Jak2) were obtained from Cell Signaling. T-

Immunoprecipitation and Western blotting

Cell lysates were immunoprecipitated with 1.5 μg of anti-Hck (Y416; Cell Signaling Tech-

Tyrosine phosphatase assay

The tyrosine phosphatase assay was performed in microglia treated with soluble and immobilized Abs using a commercially available kit (Tyrosine Phosphatase Assay System; Promega). Microglia were plated in six-well plate at 3 × 10^6 cells per well, and then soluble anti-CD45RO or control IgG2A was added for 30 min. To immobilize Abs, six-well plates were coated with Abs in PBS for 1 h at 37°C, and then microglia were seeded at 3 × 10^3 cells per well. Cells were harvested 30 min later in a phospha-
tase buffer (50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.05% 2-ME, 1% Triton X-100, and one tablet of the protease inhibitor mixture (Boehringer-Mannheim) per 10 ml of the lysis buffer) at the indicated time points. Thirty to 70 μg of protein was separated by 8 or 10% SDS-PAGE, and then transferred to polyvinylidene difluoride membrane. The blots were blocked in TBS/0.1% Tween 20 (TTBS) containing 5% nonfat milk, and then incubated with Abs (T-Hck at 1:500; β-actin at 1:5,000; all others at 1:1,000) at 4°C for 16 h. The secondary Ab was HRP-conjugated anti-mouse or anti-rabbit IgG and was used at 1:2,000 to 1:10,000 for 1 h at room temperature. Signals were developed using ECL (Pierce Biotechnology). Densitometric analysis was performed using Scion Image software.

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12,000 rpm for 5 min at 4°C. Endogenous phosphate was removed by loading the supernatants onto Sephadex-G25-containing spin columns. The sample flowthroughs were then incubated with 100 μM tyrosine phosphopeptide as a substrate for 10 min at 30°C, followed by incubation with molybdate dye/additive mixture. The amounts of free phosphate generated in the reaction were determined by measuring the absorbance of molydate:malachite green:phosphate complex.

Generation of Hck stable transfectants and [³H]thymidine incorporation
To determine whether Hck tyrosine kinase was involved in GM-CSF-induced proliferation of monocyte lineage cells, we generated Hck stable transfectants in U38 cells, a derivative of U937 cells with stably incorporated HIV-1 LTR (50). Plasmids containing wild-type (WT) and kinase-inactive (K269E) forms of human Hck (51) were obtained from Dr. T. Smithgall at the University of Pittsburgh (Pittsburgh, PA) and were subcloned into pcDNA3.1/Zeo expression vector (Invitrogen Life Technologies). The resulting constructs were used to transfect U38 cells by electroporation (Amaxa, program U-06). The stable transfectants were selected in the presence of 50 μg/ml zeocin and then expanded. Western blot analysis confirmed overexpression of the Hck protein in WT and kinase-inactive (KE) transfectants but not in control cells (not shown). Cells were incubated at 2 × 10⁶ cells per well in triplicate in 96-well plates in RPMI/10% FCS in the presence of anti-CD45RO or control IgG2a together with 10 ng/ml GM-CSF. Two days later, cultures were pulsed with [³H]thymidine for 16 h, and then cell proliferation was measured using an automated cell harvester and a beta scintillation counter.

Statistical analysis
Results shown are representative of two to five separate experiments using cells from different brain cases. For statistical analysis of the data within individual experiment, Student’s t test was performed to compare differences between the treatment and control groups. In addition, a one-way ANOVA was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (<0.05), post hoc Student-Newman-Keuls test was performed to determine which conditions were significantly different from each other.

Results
GM-CSF induces microglial proliferation
Microglial cultures were treated with medium alone (control) or GM-CSF for 3 days, and proliferation was examined by immunostaining for Ki67 (brown, nuclear) or Ki67/C68 (blue, cytoplasmic, microglial marker) double labeling. As shown in Fig. 1, Ki67⁺ microglia increase in number in GM-CSF-treated cultures. The identity of the proliferating cells is confirmed as microglia by the expression of CD68 (insets). Microglia treated with GM-CSF showed a shape change to an ameboid form. These changes in microglial cells are identical with those we reported previously (13, 14).

Effect of kinase inhibitors on GM-CSF-induced microglial proliferation
GM-CSF activates several cell signaling pathways that are implicated in cell proliferation, survival, and differentiation (16). We therefore examined pharmacological inhibitors of these pathways to determine their involvement in GM-CSF-induced microglial proliferation (Fig. 2). Microglial cultures were treated with PP2 (Src inhibitor; 1–10 μM), LY294002 (PI3K inhibitor; 1–50 μM), or U0126 (ERK MAP kinase inhibitor; 1–20 μM), and then with GM-CSF for 72 h. The Ki67 labeling index (percentage of Ki67⁺ cells) was determined as described in Materials and Methods. The concentrations of the inhibitors were chosen based on their reported IC₅₀ for their specific substrates (45, 46). The results demonstrated that PP2, LY294002, and U0126 dose-dependently inhibited microglial proliferation with IC₅₀ at ~1, 3, and 1 μM, respectively. The inhibitors were not toxic to microglia at these concentrations as determined by MTT assay (data not shown).

FIGURE 1. Human fetal microglia proliferate after stimulation with GM-CSF. Microglia were cultured with medium alone (Control; A) or with 10 ng/ml GM-CSF (B) for 3 days. Proliferation was determined by immunocytochemistry for nuclear Ki67 Ag. Microglial proliferation is minimal in control cultures, whereas GM-CSF induced Ki67 expression in ~10% of the cells (arrowheads). Inset, To confirm the identity of the proliferating cells, cultures were double labeled for Ki67 (brown in the original: nucleus) and CD68, a microglial marker (blue in the original: cytoplasm). Double-labeled cells in GM-CSF-treated cultures are shown in B (inset, arrowheads). The scale bars represent 20 μm.

Soluble but not immobilized anti-CD45RO inhibits microglial proliferation
We next examined the effect of anti-CD45 Abs on microglial proliferation. We also compared the modes of Ab administration, either as soluble form or plate-bound (immobilized) form. Microglia were treated with anti-CD45 or control Abs in the presence or absence of GM-CSF for 3 days, and then microglial proliferation was determined by Ki67 labeling index (percentage of Ki67⁺ cells). For immobilization of Abs, culture plates were first coated with anti-CD45 or normal mouse IgG2a, and then microglia were seeded onto the coated plates as described in Materials and Methods. The results showed that anti-CD45RO inhibited GM-CSF-induced microglial proliferation when the Ab was provided in soluble form (Fig. 3). Plate-bound anti-CD45RO had no effect on microglial proliferation. Abs to CD45RA (clone HI100) or CD45RB (clone MT4) had no significant effects on microglial proliferation (data not shown).

To determine whether soluble anti-CD45RO Ab acted as a CD45 agonist, we next examined tyrosine phosphatase activity in microglia treated with Abs. As shown in Fig. 3, anti-CD45RO increased tyrosine phosphatase activity in microglia when provided in soluble form. Immobilized CD45RO did not produce a significant change in tyrosine phosphatase activity (p > 0.05). Dose-response study showed 95–100% inhibition of microglial
proliferation at 5–20 μg/ml anti-CD45RO Ab (data not shown); thus all subsequent experiments were performed with soluble anti-CD45RO at 5 μg/ml. Together, our results indicate that soluble anti-CD45RO inhibits microglial proliferation by activating CD45 PTPase activity. Furthermore, CD45 tyrosine phosphatase activity is induced by soluble but not immobilized Ab.

**GM-CSF induction of cell signaling pathways in microglia**

We examined GM-CSF-treated primary human microglia by Western blot analysis for the expression of Jak/Stat proteins and kinases implicated in GM-CSF signal transduction. As shown in Fig. 4, p-Src, p-Akt (downstream of PI3K, also called protein kinase B), p-Erk MAPK, p-Jak2, p-Stat5, and p-Stat3 were detected in a time-dependent manner, whereas p-p38 MAPK or p-Stat1 were not detected (not shown). Of these, p-Src, p-Jak2, p-Stat5, and p-Stat3 appeared at 5 min, whereas p-Akt and p-Erk appeared at 10 min after GM-CSF treatment. p-Src and p-Akt persisted through the 60-min time point, whereas others were undetectable by 40 min (Figs. 4 and 6).

**Anti-CD45 inhibits microglial Src (Hck) and Akt phosphorylation induced by GM-CSF**

We next determined whether any of these pathways were inhibited by anti-CD45RO by Western blot analysis of microglial cultures with or without anti-CD45RO. As shown in Fig. 5A, anti-CD45RO inhibited p-Src. (Additional densitometric ratios (p-Src/T-Erk) from a separate microglial case were 0.33 (control), 0.72 (GM-CSF), 0.36 (plus anti-CD45RO), 0.61 (plus IgG2a).) Immunoprecipitation with anti-Hck Ab followed by immunoblotting with anti-p-Src Ab that cross-reacts with many different species of Src kinases identified the Src kinase phosphorylated by GM-CSF to be Hck, a myeloid lineage-specific Src kinase (Fig. 5B). Thus, Hck is activated by GM-CSF and inhibited by anti-CD45RO in microglia. Although anti-CD45RO did not inhibit p-Akt at 10 min (Fig. 5A), it did inhibit p-Akt at later (1- and 4-h) time points (C). (Additional densitometric ratios (p-Akt/T-Akt) from a separate microglial case were 0.79 (control), 0.85 (GM-CSF), 0.79 (plus anti-CD45RO), and 0.81 (plus IgG2a).)

Anti-CD45RO did not inhibit p-Jak2, p-Stat3, p-Erk, or p-Stat5 (Fig. 5, D and E). Together, these results demonstrate that GM-CSF activates multiple cell signaling pathways in primary human microglia and that anti-CD45RO inhibits Hck and PI3K/Akt, but not Jak2/Stat5, Stat3, or Erk MAPK pathways.

**The effect of kinase inhibitors on GM-CSF-induced microglial cell signaling**

Because the Src, PI3K, and Erk inhibitors inhibited microglial proliferation (Fig. 2), we tested the effect of these inhibitors on GM-CSF-induced microglial cell signaling by Western blot analysis. Microglial cultures were pretreated with inhibitors at 10 μM for 1 h and then stimulated with GM-CSF for 10, 30, 60, and 240 min. The results are shown in Fig. 6. PP2 (Src inhibitor) completely inhibited p-Src. PP2 also inhibited p-Akt at all time points, although the effect at 10 min was mild. p-Stat3 was also inhibited at 10 min. No effect was observed on p-Erk, p-Jak2, or p-Stat5 (Fig. 6A). LY294002 (PI3K inhibitor) completely inhibited p-Akt at all time points (Fig. 6B). The effect of kinase inhibitors on GM-CSF-induced microglial cell signaling was measured in microglia from a separate microglial case were 0.33 (control), 0.72 (GM-CSF), 0.61 (plus anti-CD45RO), and 0.81 (plus IgG2a).
Role of Hck in GM-CSF-induced proliferation of U38 cells

To directly examine the role of Hck kinase in GM-CSF-induced cell proliferation, Hck stable transfectants expressing either WT or KE Hck tyrosine kinase were generated in U38 cells as described in Materials and Methods. Cell proliferation was determined by [3H]thymidine incorporation assay following treatment with anti-CD45RO or control IgG with or without GM-CSF. The results are shown in Fig. 7. GM-CSF increased proliferation of control cells, which was inhibited by CD45 through down-regulation of JAKs (39). Further, IL-3-induced cell proliferation has been shown to be inhibited by CD45 through down-regulation of JAKs (57) and the Stat5 pathway (54–56), which we initially hypothesized to be the main pathway (Fig. 6, A and B). Together, these results indicate that PI3K/Akt is activated downstream of Hck tyrosine kinase by GM-CSF in microglia. Stat3 may also be activated downstream of Hck and PI3K/Akt, and Erk MAPK downstream of PI3K/Akt. However, other mechanisms of activation must exist for Stat3 and Erk MAPK, because their inhibition was incomplete.

Proposed GM-CSF signaling in primary human microglia

Based on the information obtained in this study and in the literature, we propose the following scheme (Fig. 8). We confirmed that the three main pathways (Jak2/Stat5, PI3K/Akt, and Erk MAPK) are activated by GM-CSF in microglia, as well. In addition, Hck tyrosine kinase is also activated and plays a major role in the induction of cell proliferation. The PI3K/Akt pathway is activated downstream of Hck probably via phosphotyrosine-containing adaptor proteins and plays a role in cell proliferation. We further propose that Stat3 and Erk might, in part, be activated downstream of Hck and PI3K/Akt and play a role in cell survival and proliferation. However, other pathways of Stat3 and Erk activation must also exist (16, 52). The main pathway of Erk MAPK activation is probably the Ras/Raf pathway, which might be activated by the adaptor proteins, Shc and growth factor receptor-bound protein 2 (Grb2) (53). Soluble anti-CD45RO activates CD45 tyrosine phosphatase activity; CD45 mainly targets Hck tyrosine kinase thereby inhibiting cell proliferation. The Jak2/Stat5 pathway appears unaffected by CD45 in microglia.

Discussion

In this study, we investigated GM-CSF signal transduction in primary human microglia and the mechanisms involved in CD45-mediated inhibition of microglial proliferation. We found that stimulation of CD45 with a specific Ab inhibited GM-CSF signal transduction and cell proliferation in human microglia by dephosphorylating Hck tyrosine kinase. One of the main pathways activated by GM-CSF in monocytes and macrophages is the Jak2/Stat5 pathway (54–56), which we initially hypothesized to be the CD45 substrate in microglia. Jak2 has been shown to be both necessary and sufficient for βc-induced cell proliferation (57) and the JAKs are known substrates for CD45 in mast cells and B cells (39, 58). Furthermore, IL-3-induced cell proliferation has been shown to be inhibited by CD45 through down-regulation of JAKs (39).
Nevertheless, our results in microglia demonstrate that the Jak2/Stat5 pathway is not inhibited by CD45 and is probably not involved in GM-CSF-induced cell proliferation. Western blot analyses and inhibitor experiments also suggested that Jak2 and Stat5 are activated independent of the Src kinase (Hck), PI3K, or Ras/Erk MAPK pathways in microglia. Interestingly, Src homology 2 domain-containing PTPase-1 has also been implicated in the down-regulation of GM-CSF-induced macrophage proliferation, in a manner independent of Jak2, Stat5, or MAPKs (59). Moreover, analysis of GM-CSF receptor mutants revealed nonredundant functions for the different regions of the βc chain; in particular, the tyrosine residue essential for cell proliferation (Y577) has been shown to be located distal to the membrane-proximal region that is essential for Jak2 activation (60). Together, these data strongly support that the Jak2/Stat5 pathway is not involved in GM-CSF-induced microglial proliferation.

Instead, the Src kinases appear to play an important role in GM-CSF-mediated microglial proliferation. Previous studies have indeed suggested a crucial role played by the Src kinases in βc signal transduction, particularly with respect to their role in the activation of Stat, Ras/MAPK, as well as PI3K pathways (61). Furthermore, Lyn has been shown to bind to βc-derived peptides and induce tyrosine phosphorylation of the βc chain (62). Our experiments investigating the relationship between the Src (Hck) and PI3K pathways in GM-CSF-stimulated microglia support that PI3K/Akt was activated downstream of Hck, and that both are involved in microglial proliferation. There is no apparent consensus binding site for PI3K in the βc chain, and adaptor molecules such as Src homology 2 domain-containing PTPase-2 and Grb2 have been proposed to recruit PI3K to the receptor (16). The demonstration that Src kinases are involved in the activation of PI3K in GM-CSF signal transduction in microglia is similar to that shown in human neutrophils (63) and suggests that, in these cells, Src kinases may be complexed with the adaptor proteins and may activate PI3K (61).

We demonstrated that Hck is necessary for GM-CSF-induced cell proliferation in U38 cells stably overexpressing KE Hck. Although GM-CSF readily increased cell proliferation of control (empty vector) or WT Hck-expressing cells, it had no effect on KE-expressing cells. Moreover, anti-CD45RO abrogated GM-CSF-induced proliferation of U38 cells, as it did in microglia. Together, these results indicate that Hck tyrosine kinase is a crucial player in GM-CSF-mediated macrophage and microglial proliferation. Indeed, Smithgall and colleagues (64) have demonstrated a role for Hck in the survival/proliferation of a GM-CSF-dependent human macrophage cell line, TF-1. TF-1 cells expressing HIV-1 accessory protein Nef show phosphorylation of Hck and...
proliferate without GM-CSF. Interestingly, in TF-1 cells, Stat3 was shown to be a downstream effector of Nef-activated Hck (64). Stat3 is a transcription factor activated by cytokines, growth factors, and IFNs belonging to the gp130 receptor family that is involved in the induction of growth-promoting genes (65, 66). The mechanism of Stat3 activation by GM-CSF is not clear, but the Src kinases have been implicated in βc-mediated Stat protein activation (61). It has been also suggested that the nature of Stat activation depends on the cell type rather than the nature of Jak proteins activated (61). In chick chorioallantoic membrane, GM-CSF stimulates angiogenesis by activating the Jak2/Stat3 pathway (52). In microglia, GM-CSF induced phosphorylation of Stat3, which was partially inhibited by LY294002. These results suggest that Stat3 may act downstream of PI3K/Akt to increase the survival and proliferation of GM-CSF-responsive cells.

MAPK pathways are also known to be activated following GM-CSF receptor engagement, and this has been shown previously in rodent microglia (57). The major pathway responsible for activation of Erk MAPK in microglia is probably the Ras/Raf pathway, which, in turn, is activated through adaptor proteins such as Shc and Grb2 (16, 53, 67). In human microglia, although anti-CD45 or PP2 had no effect, LY294002 slightly inhibited p-Erk, suggesting its activation is downstream of PI3K/Akt. Despite the fact that U0126 (Erk inhibitor) failed to inhibit several GM-CSF signal transduction pathways, U0126 abrogated microglial proliferation. Transition from G0 to G1 and subsequent progression through the cell cycle involves activation of several cell-cycle-dependent kinases through the coordinated regulation of several signal transduction pathways. The sum of all necessary pathways activated by GM-CSF would push microglial cells through the cell cycle, whereas blocking any one of the pathways (via Erk, for example) could efficiently inhibit cell proliferation.

Our results demonstrate that the inhibition of proliferation by anti-CD45 correlated with whether the Ab induced tyrosine phosphatase activity. We found that although soluble anti-CD45 induced PTPase activity and inhibited microglial proliferation, immobilized Ab failed to induce tyrosine phosphatase activity and did not inhibit microglial proliferation. These results indicate that Ab-mediated cross-linking of the CD45 molecule was not the mechanism by which anti-CD45 mAb activated CD45. Our results rather suggest that cross-linking of CD45 might actually inhibit CD45 activity. On review of the literature, we find this notion corroborated by other studies. The reported proinflammatory activity (e.g., increase in cytokine production) of anti-CD45 mAb on monocytes and macrophages was induced by immobilized Abs (68, 69), whereas macrophage inhibitory activity was induced by soluble Abs (42, 43). These results call for caution in considering the Ab-based modulation of CD45 activity for therapy.

How the anti-CD45 mAb modulates CD45 activity is unclear, but it has been suggested that CD45 may spontaneously form dimers in cells, resulting in the suppression of CD45 PTPase activity (70–72). Furthermore, dimerization is shown to be isoform-dependent, with CD45R0 more efficiently dimerizing than the larger isoforms due to the absence of sialylation and O-glycosylation of the alternatively spliced exons (73, 74). Interestingly, we find that Abs against CD45RA or CD45RB are ineffective in inhibiting microglial proliferation (not shown). Based on the available information, we speculate that CD45 protein exists as dimers on microglia and the soluble anti-CD45R0 disrupts the dimerization process releasing suppressed phosphatase activity. If disruption of dimerization is the basis for Ab activity, we expect anti-CD45R0 to be most effective. Studies investigating isoform-specific Ab function are rare, but one such study reported suppression of human lymphocyte activation by anti-CD45R0 (clone UCHL-1) (75).

Our study adds to the growing list of literature that demonstrates the diverse roles played by CD45. Because activated microglial cells express robust amounts of CD45 and microglial activation products are implicated in neuronal damage, CD45 may present as a new molecular target for treating CNS diseases, as has been suggested for cancer, transplantation, and autoimmune (76, 77). Future studies are necessary to address the in vivo efficacy of such therapy as well as the effect of CD45 signaling in other CD45+ cells (i.e., inflammatory cells) in the CNS.

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Disclosures
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