Expression of the HGF receptor c-met by macrophages in experimental autoimmune encephalomyelitis

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ABSTRACT

Hepatocyte growth factor (HGF) is a pleiotropic cytokine able to evoke a wide array of cellular responses including proliferation, migration, and survival through activation of its receptor c-met. Various types of leukocytes have been described to express c-met suggesting that HGF/c-met signaling may directly influence leukocyte responses in inflammation. We have investigated the HGF/c-met pathway in experimental autoimmune encephalomyelitis (EAE), a commonly used mouse model of multiple sclerosis (MS), in which macrophages play a dual role, contributing directly to CNS damage at disease onset but promoting recovery during remission by removing myelin debris.

Here we show that during EAE both HGF and c-met are expressed in the CNS and that c-met is activated. We subsequently demonstrate that c-met is primarily expressed in inflammatory lesions by macrophages and a small number of dendritic cells (DCs) and oligodendrocyte progenitor cells (OPCs) but not by microglia or T cells. Complementary *in vitro* experiments show that only LPS and TNFα, but not IL-6, IL-10 or IL-13, are able to induce c-met expression in macrophages. In addition, using TNF signaling deficient macrophages we demonstrate that LPS and TNFα induce c-met through distinct pathways. Furthermore, TNFα- and LPS-induced c-met is functional because treatment of macrophages with recombinant HGF results in rapid phosphorylation of c-met. Interestingly, HGF/c-met signaling does not modulate cytokine expression, phagocytosis or antigen presentation but promotes proliferation of activated macrophages. Taken together, our data indicate a pro-inflammatory role for the HGF/c-met pathway in EAE rather than a role in the initiation of repair mechanisms.

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease of the CNS associated with demyelination, inflammation and axonal loss (Franklin and Ffrench-Constant 2008; Hemmer et al. 2002; Wekerle 2008). The main inflammatory effector cells are $CD4^+$ T cells (Bynoe et al. 2007; Platten and Steinman 2005; Wekerle 2008), which are initially primed in peripheral lymphoid tissues mainly by antigen presenting dendritic cells (DCs). An additional local T cell re-activation phase mediated by CNS-associated antigen presenting cells (APCs), such as microglia, macrophages and DCs, is thought to be essential for the disease to develop (Becher et al. 2006; Platten and Steinman 2005). In addition activated microglia and CNS-infiltrating peripheral macrophages contribute directly to CNS damage through the production of pro-inflammatory cytokines, matrix metalloproteinases, glutamate, and free radicals (Hemmer et al. 2002; Piani et al. 1991; Piani et al. 1992; Platten and Steinman 2005). Important mediators of the cellular inflammatory response during the initiation and peak phase of the disease are pro-inflammatory cytokines such as TNFα, IFNγ and IL-17, whereas recovery from EAE is initiated by a shift to anti-inflammatory cytokines such as IL-4, IL-10 and TGFβ (Hemmer et al. 2002; Platten and Steinman 2005). During the remission phase of MS, macrophages and microglia are thought to play a more beneficial role by removing cellular and myelin debris, a process essential for remyelination to occur because myelin debris inhibits oligodendrocyte progenitor cell (OPC) migration and differentiation (Franklin 2002; Franklin and Ffrench-Constant 2008).

Hepatocyte growth factor (HGF), also known as scatter factor, was discovered by virtue of its growth inducing properties of hepatocytes (Nakamura et al. 1986) and its ability to induces scattering of epithelial cells (Stoker et al. 1987). HGF, a disulphide-

linked α-and β -chain heterodimer with homology to the proteinases of the plasminogen family, mediates multiple biological responses by activation of its cognate tyrosine kinase receptor c-met (Benvenuti and Comoglio 2007; Corso et al. 2005). C-met is a tyrosine kinase receptor consisting of an exclusively extracellular α-chain and a disulphide-linked trans-membrane β-chain that contains a multifunctional docking site able to interact with several cytoplasmic signal transducers such as the scaffolding adaptor Gab1. Gab1 is crucial in many aspects of c-met signaling, binding various signal-relay molecules such as phosphatase Shp2, PI3K, phospholipase C and Crk which act upstream of Ras and Raf, two components of the ERK/MAPK pathway (Benvenuti and Comoglio 2007; Bolanos-Garcia 2005; Corso et al. 2005).

C-met activation by HGF is able to evoke a wide array of cellular responses including proliferation, migration, scattering, survival, and branched morphogenesis depending on cell type and context (Benvenuti and Comoglio 2007; Corso et al. 2005). Notably, HGF c-met signaling may play a role in the modulation of disease (Skibinski 2003). In many inflammatory diseases, including bacterial meningitis (Nayeri et al. 2000), and multiple sclerosis (Kern et al. 2001; Tsuboi et al. 2002), serum and/or local tissue concentrations of HGF are increased. In general, HGF is thought to play a beneficial role in disease, stimulating repair mechanisms of damaged tissues. However, various types of leukocytes including macrophages (Chen et al. 1996; Galimi et al. 2001), monocytes (Beilmann et al. 1997; Jiang et al. 2001), dendritic cells (Ovali et al. 2000; Rutella et al. 2006), B cells (van der Voort et al. 2000), and T cells (Adams et al. 1994) have been described to either express c-met and/or show cellular responses to HGF indicating possible direct effects of HGF on leukocytes in inflammation. Especially for monocytes the evidence is compelling with several studies describing the induction of c-met and HGF in human monocytes in response to various

inflammatory cytokines. However, the function of HGF/c-met signaling in monocytes/macrophages is at present a matter of debate with, functions ranging from migration to differentiation reported (Beilmann et al. 1997; Beilmann et al. 2000; Chen et al. 1996; Galimi et al. 2001; Jiang et al. 2001). Although HGF/c-met signaling may directly influence leukocyte responses in inflammation and autoimmune diseases, the mechanisms involved remain at present poorly defined.

We have investigated the HGF/c-met pathway in experimental autoimmune encephalomyelitis (EAE), a well-established mouse model of multiple sclerosis. In a previous report we showed that microglia produce HGF in response to TGFβ (Lalive et al. 2005). Now we show that both HGF and the HGF receptor c-met are expressed in the CNS of EAE diseased mice. Flow cytometric and immunohistochemical studies indicate that c-met is primarily expressed by $CD11b^+$ macrophages and to some extent by oligodendrocyte progenitor cells (OPCs) but not by T cells or dendritic cells (DCs). Complementary *in vitro* experiments indicated that "classical" but not "alternative" activation of macrophages induces c-met expression. Furthermore, we show that LPS and TNFα induce c-met through distinct pathways. Functionally HGF/c-met signaling does not appear to be involved in cytokine expression, phagocytosis or antigen presentation but rather promotes proliferation of activated macrophages.

MATERIALS AND METHODS

Mice

Seven to eight week old C57Bl/6 female mice were purchased from Harlan (the Netherlands). TNFαko mice (B6.129-*TNF* ^{tm1Ljo}; (Marino et al. 1997) were obtained from the Ludwig Institute for Cancer Research. TNFR1ko mice (Tnfrsf1atm1Blt; (Rothe et al. 1993) and TNFR2ko mice (Tnfrsf1btm1Imx; unpublished) were a kind gift from Mathias Heikenwälder, University Hospital Zurich, Switzerland.

Reagents

Cytokines, growth factors and chemicals were obtained from the following sources. Roche Diagnostics: mTNFα and mIFNγ. Sigma: LPS-B5 and -B8. PeproTech: rmIL-1β, hIL-2, mIL-3, rmIL-4, mIL-6, mL-12, rmIL-13, rmTNFβ, rhTGFβ2, mMip-1α, mRANTES, M-CSF, and rhHGF. Pharmingen: hIL-8 and rmIL-10. R&D systems: rhTGFβ1 and 3, and IL-17. Calbiochem: L-NMA. Sigma: PMA. Mouse HGF Duoset ELISA kit was purchased from R&D systems and used according to the manufacturer's instructions.

Antibodies to c-met were obtained from R&D systems and anti-c-met PY$^{1230/1234/1235}$ from Biosource. Anti-NG2 and PDGF-R (CD140a) were purchased from Chemicon. Antibodies to CD4 (RM4-5, Biotin; RM4-4, FITC), CD8 (Biotin, FITC), CD11c (HL3, Biotin), CD11b (M1/70, APC, Biotin), CD16/32 (Fc block), CD45 (APC), CD80 (16-10A1, FITC), CD86 (GL1, FITC), MHC class II (M5, PE), were obtained from BD Pharmingen and F4/80 (FITC) from Serotec. The secondary antibody swine anti-goat-PE was purchased from CALTAG and anti-goat HRP and anti-rabbit HRP from Pierce. Anti-rat Alexa 488 was obtained from Molecular Probes and Streptavidin-FITC from BD Pharmingen

EAE induction

C57BL/6 mice were injected with 100 µg MOG35-55 (Anawa, Switzerland) in 200 µl PBS/CFA (DIFCO, USA) (1:1) subcutaneously on the right flank and with 300 ng Pertussis toxin (List Biological Laboratories, USA) intraperitoneally (i.p.) on day 0, followed by a boost of 300 ng Pertussis Toxin at day 2 and 100 µg MOG35 in CFA into the left flank on day 7. The scoring of clinical symptoms was performed as described previously (Eugster et al. 1999).

Isolation of CNS-mononuclear cells

CNS-mononuclear cells were isolated as described previously (Suter et al. 2003). Briefly, brains and spinal cords of animals perfused with Hanks' balanced salt solution (HBSS) were minced with a scalpel blade and digested for 30' at 37°C in HBSS containing 50 µg/ml DNase I and 100 µg/ml Collagenase/Dispase (Roche). The digestion was quenched on ice and passed through a 100 µm Nylon mesh (BD Biosciences) and centrifuged after which the pellet re-suspended in 30% Percoll (Sigma). The gradient was centrifuged at 29000 x g for 30 min at 4°C (Kontron Instruments, Germany). The top layer containing myelin was removed by aspiration, and the interphase containing mononuclear cells was collected, diluted threefold with HBSS and collected by centrifugation at 300 x g.

Primary cells and cell lines

For the isolation of splenocytes, spleens were removed and mashed through a 100 µm cell strainer followed by lysis of erythrocytes in ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2).

Peritoneal exudate cells (PECs) were recovered 2 days (optimal for a high recovery of macrophages) after i.p. injection of 1 ml 3% Brewer thioglycollate medium (Sigma). The peritoneal cavity was flushed with 10 ml HBSS/1% bovine serum albumin (BSA)/15 mM Ethylenediaminetetraacetic acid (EDTA).

Primary microglial cells were obtained from a co-culture of glial cells as described previously (Lalive et al. 2005). Briefly, pups were decapitated and whole brains were homogenized mechanically. Brain homogenates were seeded in DMEM (Gibco) containing 10% fetal calf serum (FCS) (PAA Laboratories GmbH, Austria), 2 mM N-Acetyl-L-alanyl-L-glutamine (L-GLU) (Biochrom AG, Germany) and 20 µg/ml gentamycin (Sigma) and cultured at 37°C, 8% CO_2. On day 14 microglia were isolated from the glial feeder layer by mechanical shaking at 1,400 rpm for 25 min.

Primary oligodendrocyte progenitor cells (OPCs) were isolated as described previously (Lalive et al. 2005). Briefly, neonatal forebrain cells were harvested from 1-2 day old neonates C57/BL6 mice by dissociation of cortices in Papain (Sigma) and grown in DMEM supplemented with 10% horse serum (HS) (Gibco) on poly-D-lysine (Sigma)-coated cell culture flasks. After 10 days the loosely attached OPCs were separated from the glial feeder layer by 16 h of mechanical shaking at 210 rpm, 37°C.

Bone marrow-derived dendritic cells (BM-DC) prepared as described previously (Landmann et al. 2001).

Bone marrow-derived macrophages (BMM) were prepared from bone marrow cells isolated from the femur and tibia. Bones were flushed with HBSS and the cell suspension was forced through a 70 µm mesh. Collected cells were re-suspended in complete macrophage medium containing DMEM, 30% L929 cell-conditioned medium (source of M-CSF), 20% HS, 10 mM HEPES (Gibco) and 10 mM L-GLU and cultured at 37°C, 5% CO2. After 7 days virtually 100% of the cells expressed the macrophage markers CD11b and F4/80. The cells were harvested by scraping on ice and frozen in 90% FCS and 10% DMSO at a density of 5×10^6-10×10^6 cells/ml. Thawed cells were cultured in either DMEM containing 10% FCS, 2 mM L-GLU and 20 µg/ml gentamicin or the same medium without serum.

OLI-neu cells (kindly provided by Dr. J. Trotter) were cultured in SATO-medium as described previously (Lalive et al. 2005).

To determine c-met expression, all cell types were seeded in 6-well culture plates at 1.0-1.5×10^6 cells/well and treated with cytokines for 48 h prior to flow cytometric and/or real time PCR analysis.

Flow cytometric analysis

Cells were re-suspended in FACS buffer containing 2% FCS, 5 mM EDTA, 0.01% NaN_3 in phosphate buffered saline (PBS). Prior to staining with the appropriate antibodies, Fc receptors were blocked by incubation with anti mouse CD16/32 (Fc-block, BD Pharmingen). 7-Amino-actinomycin D (7-AAD) was used to exclude non-viable cells. All flow cytometric data was obtained with a CyFlow flowcytometer (Partec, Germany). Data analysis was performed with the freeware program WinMDI.

c-met immunoprecipitation and Western blotting

Immunoprecipitation of c-met was based upon a previously published method (Moransard et al. 2003) and extensively tested on mouse liver samples and the HEPA 1-6 cell line (data not shown). Cultured cells were scraped on ice in lysis buffer containing 1% Nonidet P 40 (NP-40, Igepal CA 630, Fluka), 50 mM Tris, 150 mM NaCl, 5 mM EDTA and a cocktail of proteinase inhibitors (Complete Mini, Roche). Spinal cords isolated from EAE diseased mice were homogenized on ice with a douncer in the same buffer (NP-40 was added after homogenization). Homogenates and lysates were cleared from insoluble material by centrifugation and the supernatants were incubated with anti-c-met antibody or isotype control antibody followed by incubation with Protein G Dynabeads (Invitrogen). The beads were washed with lysis buffer containing 0.5% NP-40 followed by incubation with an equal volume of Laemmli buffer at 80°C prior to Western blotting for c-met and

phosphorylated c-met. Scanned images of Western blots were quantified using ImageJ (Freeware, National Institute of Health, USA)

Immunohistochemistry

Mice were CO_2 anesthetized and perfused with 25 ml Ringer solution (Braun Medical AG, Switzerland). Immunohistochemistry was performed on 10-15 µm frozen, 4% PFA- or methanol-fixed longitudinal sections of spinal cord. Sections were thawed, fixed and blocked (5% FCS, 0.01% triton, PBS) prior to incubation with primary antibody 1/200 in block buffer for 24 h. After washes with PBS, sections were incubated with the appropriate secondary antibody and/or Streptavidin-FITC (BD Pharmingen) in block buffer for 2 h. After counterstaining with 4'6-diamidino-2-phenylindole (DAPI, Roche) sections were mounted in Mowiol embedding medium (Mowiol 4-88, Calbiochem) containing 0.1% 1,4-Diazabicyclo-82.2.29-octan (DABCO, Fluka).

RNA isolation and real time PCR

Whole-cell RNA from cultured cells was extracted using the NucleoSpin-RNA II kit (Macherey-Nagel, Switzerland). RNA from mouse tissues was extracted by homogenisation in TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using random hexamers and AMV reverse transcriptase (Promega). The cDNA equivalent to 50 ng of total RNA was PCR-amplified in an ABI PRISM 7700 detection system (PE-Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and quantified using the $2^{-\Delta\Delta CT}$ method using 18s rRNA as a housekeeping gene. Relative RNA levels are expressed as x-fold variations compared to control. Primers and probes for Taqman analysis for IL-1β, IL-6, IL-10, TNFα, and iNOS were purchased from Applied Biosystems. Primers and probes for c-met (forward primer 5'-GCA TGT CAG CAT CGC TCA A-

3', probe 5'-FAM-TTC AGA GAT CAT CTG CTG CAC TAC TC-3'-TAMRA , reverse primer 5'-GGC CCA GCT GTT TCA GTG AA-3') and HGF (forward primer 5'-TGG TGG CCC ACT CAT TTG T-3' , probe 5'-FAM-AAA TGA GAA TGG TTC TTG GTC TCA TT-3'-TAMRA, reverse primer 5'-ATT TGG GAT GGC ACA TCC A-3') were purchased from Microsynth, Switzerland.

Nitrate test (Griess test)

Medium aliquots (in triplicate) from LPS or LPS + IFNγ treated BMM cultures co-treated with or without 50 ng/ml HGF were incubated in a 96 well plate with 0.04% Napthylethylenediamine-dichloride (Sigma), 0.4% Sulfanylamide (Sigma) and 1.2% H_3PO_4 at RT for 10 minutes. After determination of the absorption at 540 nm on a plate reader, the concentration of nitrite in the medium was calculated using a standard curve of serial dilutions from a 512 μM $NaNO_2$ stock solution.

MTT cell viability assay

BMM were seeded in a 96 well plate and treated with LPS for 16 h prior to the addition of 50 ng/ml HGF or carrier only, followed 1h later by the addition of camptothecin (Sigma) or staurosporine (Sigma) at various concentrations. 20 μl of a 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution was added followed by incubation at 37°C for 5 h. After removal of the medium and the addition of DMSO, the absorbance was measured on a plate reader at 550 nm.

Phagocytosis assay

BMM were grown on 6 well plates and treated with LPS or left untreated. 50 ng/ml HGF was added 1 h prior to addition of 1 μm PE-labelled carboxylated polystyrene beads (Polysciences USA) for 30 minutes. The cells were extensively washed with

FACS buffer, harvested by scraping on ice and re-suspended in FACS buffer for flow cytometric analysis.

Proliferation assay

BMM at various concentrations were seeded in a 96 well plate and treated with LPS for 16 h before the addition of 5 μCi/ml ^{3}H-thymidine (GE Healthcare) together with or without 50 ng/ml HGF. After incubate for 16 h at 37°C the cells were harvested directly onto a glass fiber filter membrane using a 96-well plate harvester. The filters were dried at 60°C, sealed in a plastic bag containing scintillation fluid (PerkinElmer) and measured in a beta-counter (PerkinElmer).

RESULTS

HGF, c-met expression and c-met phosphorylation are increased in the CNS of mice with EAE.

HGF mRNA expression was examined by quantitative RT-PCR in the spinal cord of MOG35-55 immunized mice at different EAE disease stages. On day 10 post-immunization (p.i.), before clinical signs were present (preclin), HGF mRNA levels were comparable to those in naïve mice, whereas at the peak of disease (d16-18 p.i., average score 3.33±0.17), HGF mRNA levels were significantly elevated (688%±115). In the remission phase (d31 p.i., average score 1.75±0.11), HGF mRNA levels were still elevated although significantly reduced compared to the levels at peak disease (380%±140) (Fig. 1A).

Measurement of HGF protein levels in the spinal cord (Fig. 1B) and serum (Fig. 1C) with ELISA showed that at the onset of disease (d14 p.i., average score 0.25±0.14). HGF levels in spinal cord (1.76±0.29 ng/mg protein) and serum (2.72±0.26 ng/ml) were comparable to those found in naïve mice (2.27±0.28 ng/mg protein, 2.98±0.37 ng/ml respectively). In contrast, HGF amounts in mice at the peak of disease (day 16-18 p.i., average score 3.10±0.19) were significantly elevated in both spinal cord (6.57±0.97 ng/mg protein) and serum (4.57±0.33 ng/ml), with the largest increase found in the spinal cord (2.9 fold). At the remission phase (day 44 p.i., average score 2.20±0.12), serum HGF levels (3.07±0.45 ng/ml) returned to values found in naïve mice, whereas HGF amounts in spinal cord (3.92±0.14 ng/mg protein) remained slightly elevated. Thus, the expression of HGF mRNA in the CNS and the higher amounts of HGF protein in the CNS compared to serum indicate a source of HGF within the CNS rather than in the periphery, as we have suggested in an earlier report (Lalive et al. 2005).

The observed increase in HGF levels led us to examine the expression of the HGF receptor c-met in the spinal cord at the different disease stages. Quantitative RT-PCR showed that c-met mRNA was elevated at both peak (day 18 p.i.) and remission (day 31 p.i.) but not at the preclinical stage (day 10 p.i.) (Fig. 1D). Analysis of c-met protein in the spinal cord at the different disease stages by immuno-precipitation and Western blotting showed that c-met protein amounts were elevated at all disease stages (Fig. 1E lower panel) with significant increases at peak and remission (Fig. 1F). Importantly, western blotting with anti $pY^{1230/1234/1235}$ c-met antibody (detects activated c-met) showed that c-met phosphorylation is significantly increased at all disease stages with phosphorylation reaching a maximum at peak disease (Fig. 1E, upper panel and Fig. 1G). Taken together, these data show that the increase in HGF and c-met, as well as the activation of c-met, correlate with the peak phase of EAE, suggesting a pro-inflammatory role for HGF signalling in EAE.

The HGF receptor c-met is predominantly expressed by macrophages in EAE.

Various types of leukocytes, glia and neurons are able to express c-met (Birchmeier and Gherardi 1998; Kilpatrick et al. 2000; Shimazaki et al. 2003; Tyndall and Walikonis 2006; Yan and Rivkees 2002). Therefore we determined which cells express c-met in the spinal cord during the course of EAE by immunohistochemistry. Strikingly, at peak disease c-met expression was confined to large clusters of infiltrating leukocytes, easily identified by DAPI nuclear stain (Fig. 2A-E). Co-staining for c-met and markers for oligodendrocyte precursor cells (OPCs), macrophages, dendritic cells (DCs) and T cells showed that at peak EAE c-met is mainly found on $CD11b^+$ macrophages although not all $CD11b^+$ cells expressed c-met (Fig. 2C). C-met expression was also found on a small number of $CD11c^+$ dendritic

cells (Fig. 2D) but never on $CD4^+$ T cells (Fig. 2E). In addition, a modest number of $NG2^+$ cells expressed c-met (Fig. 2A), whereas $PDGFR^+$ cells, although found in close proximity to c-met^+ clusters of cells, did not appear to express c-met themselves (Fig. 2B). Both NG2 and PDGFR are commonly used markers for oligodendrocyte progenitor cells (OPCs). At the remission phase, c-met^+ cells were no longer observed although small numbers of $CD11b^+$ cells were still detectable (Fig 2F).

In addition, flow cytometric analysis was used to determine c-met expression on leukocytes in the periphery during EAE. Clearly, a small population of c-met^+ cells was present in the spleen at peak disease that was not present at the onset or remission phase of disease or in naïve mice (Fig. 3A). Further analysis of splenocytes isolated from peak EAE diseased animals showed that approximately 43% of the $CD11b^+$ cells are c-met^+ whereas $CD4^+$ and $CD8^+$ T cells did not express c-met (Fig. 3B and C). Collectively, these data indicate that c-met is mainly expressed by $CD11b^+$ macrophages in the CNS and spleen at peak EAE disease.

Activated macrophages and OPCs, but not microglia or DCs, express c-met.

Our observations indicated that the majority of c-met^+ cells in the spinal cord are $CD11b^+$ macrophages. To assess if c-met is in general expressed by macrophages we determined c-met expression in bone marrow-derived (BMM) and peritoneal macrophages by flow cytometry. Interestingly, although quiescent BMM *in vitro* did not express c-met, treatment with LPS or TNFα led to robust expression (Fig. 4F) indicating that only activated macrophages express c-met. In good agreement, peritoneal exudate cells (PECS), isolated 48h after induction of sterile peritonitis by thioglycollate injection, contained a population of c-met^+ cells expressing the macrophage markers CD11b and F4/80 (Fig. 4E).

The $CD11b^+/c\text{-}met^+$ cells we observed in the spinal cord at peak EAE may originate from CNS-resident microglia or blood-derived macrophages. *In vitro* however, both BV2 cells, a well-established microglial cell line (Bocchini et al. 1992), and primary microglia did not express c-met even after activation with LPS or TNFα (Fig. 4A and 3B).

In addition to $CD11b^+$ macrophages, a small number of DCs and OPCs expressed c-met at peak EAE in the spinal cord. However, *in vitro*, both untreated and LPS-activated murine bone marrow derived DCs (BMDCs) failed to express c-met (Fig. 4C). In contrast, OLI-neu cells, a well-established OPC cell line (Jung et al. 1995) displayed a modest c-met expression upon TNFα + IFNγ treatment (Fig. 4D). Treatment with only TNFα had no effect on c-met expression.

Thus, *in vitro*, c-met expression can be induced in macrophages and to a limited degree in OPCs, but not in microglia and DCs. Moreover, *in vivo*, inflammatory conditions induce c-met expression in peritoneal macrophages.

LPS and TNFα induce c-met expression in macrophages through independent pathways in a dose and time-dependent manner.

The robust expression of c-met by BMM led us to focus our efforts on the regulation of c-met expression in macrophages. First, we confirmed our flow cytometric observations by c-met immunoprecipitation from cultured BMM treated with TNFα or LPS. Both stimuli induced c-met protein expression in otherwise c-met negative BMM (Fig. 5A). Importantly, treatment of LPS-activated BMM with recombinant HGF triggered phosphorylation of c-met indicating receptor activation (Fig. 5B). Furthermore, a time course study showed that phosphorylation of c-met is rapid, occurring within 5 minutes of HGF addition (data not shown).

Dose-response studies showed that 25 ng/ml TNFα or 10 ng/ml LPS are sufficient to trigger maximum c-met expression (Fig. 5C). A time course study showed that LPS and TNFα-induced c-met expression is remarkably slow; maximum receptor expression was only reached after 40h (Fig. 5D). Notably, c-met mRNA expression in response to LPS also required several hours and expression increased progressively over time (Fig. 5E). Thus both TNFα and LPS induce expression of functional c-met in a time and dose dependent manner.

Since LPS-activated macrophages produce large quantities of TNFα (Fujihara et al. 2003), LPS-induced c-met expression could potentially result from autocrine TNFα signaling. Quantitative RT-PCR showed that indeed TNFα mRNA expression preceded c-met mRNA expression by several hours (Fig. 5E). To further investigate this possibility, we treated BMM from TNFα receptor 1 (TNFR1ko), TNFα receptor 2 (TNFR2ko) and TNFα (TNFαko) deficient animals with either LPS or TNFα. Immunoprecipitation of c-met showed that TNFα did not induce c-met expression in TNFR1ko BMM but did trigger c-met expression in TNFR2ko and TNFαko BMM. Thus TNFα induces c-met expression in BMM through TNFR1 signaling. Importantly however, LPS was able to induce c-met protein expression in TNFR1ko, TNFR2ko and TNFαko BMM, clearly showing that LPS-induced c-met expression in macrophages does not require TNFα signaling (Fig. 5F).

Alternative activation of macrophages does not induce c-met expression and does not prevent or alter c-met expression induced by TNFα or LPS.

To investigate which other stimuli besides TNFα and LPS are able to induce c-met expression, we treated BMM cultures with different cytokines, chemokines and chemicals (Supplementary Table 1). Remarkably of all the stimuli tested, only TNFα

and LPS induced c-met expression whereas other pro-inflammatory cytokines important in the progression of EAE such as IL-6, IL-12 and IL-17 do not (Fig. 6A). Notably, co-treating BMM with IFNγ did not enhance LPS- (Fig. 6A) or TNFα-induced (data not shown) c-met expression. Importantly, alternative activation of macrophages with IL-4, IL-13 or IL-10 did not induce c-met expression either (Fig. 6A). Potentially, de-activation (or a change in activation status) of classically activated BMM could alter c-met expression or, *vice versa*, alternative activation of BMM could prevent LPS-induced c-met expression. However, further flow cytometric analysis showed that IL-10 or IL-13 treatment does not alter c-met expression induced in response to LPS (Fig. 6B) and that BMM activated with IL-4, IL-10, or IL-13 still respond to LPS with an increase in c-met expression (Fig. 6C). In contrast, treatment of LPS-activated BMM with recombinant HGF did show that c-met in macrophages can be down regulated by its natural ligand (Fig. 6B). Quantitative analysis of the mean fluorescence intensity (MFI) of c-met expression showed that LPS and TNFα treatment induce a comparable expression (207%±19 and 200%±48, respectively), that IL-10 does not down-regulate LPS-induced c-met (192%±31) and that HGF-treatment leads to a small but significant reduction in LPS-induced c-met expression (173%±12, $p=0.01$). Collectively, these data indicate that only the classic activators of macrophages induce c-met expression.

HGF induces proliferation of LPS-activated macrophages but does not modulate classic macrophage functions.

We next assessed whether HGF can modulate classical macrophage functions. Cytokine secretion and NO production by macrophages are important in the progression of EAE and are the main cause of myelin and axonal damage (Hendriks

et al. 2005). Quantitative RT-PCR analysis of the transcript levels of iNOS, c-met, and the cytokines IL-1β, IL-10, and TNFα in LPS-activated BMM after 1, 7 or 16h HGF treatment (Fig. 7A) showed that HGF does not significantly alter the expression of these transcripts. IL-6 showed a trend towards up-regulation although not significant. In addition, transcript levels of the cytokine receptors IL-1R, IL-2R, IL-10R and the anti-apoptotic proteins Bcl-2 and Bcl-xL were unaffected by HGF treatment (data not shown). Furthermore, HGF did not alter the amount of nitrite produced by LPS-activated BMM (Fig. 7B). Notably, the addition of IFNγ together with LPS significantly increased the amount of nitrite produced but also in this setup HGF did not alter nitrite production (Fig. 7B).

In addition to their role in innate immunity, macrophages can contribute to adaptive immune responses through the presentation of antigenic peptides on MHC molecules (Becher et al. 2006; Hume 2008). Flow cytometric analysis revealed that only a minority of LPS-activated BMM expresses MHC class II molecules and that the co-stimulatory molecules CD80 and CD86 are expressed at very low levels (Fig. 7C, upper panels). Notably, treatment with HGF did not alter the expression levels of these molecules or the levels of CD45, CD11b or F4/80 (Fig. 7C, lower panels).

Macrophages are essential in the recovery from EAE removing myelin debris by phagocytosis, a process critical for remyelination (Franklin and Ffrench-Constant 2008). We assessed the effect of HGF on the phagocytic capacity of macrophages by incubating BMM with PE-labeled polystyrene beads followed by flow cytometric analysis. Although LPS treatment dramatically enhanced the phagocytic capacity of BMM, 4, 16 or 40h of HGF treatment did not influence phagocytosis (Fig. 7D).

HGF can elicit diverse responses in a wide variety of cells including proliferation, migration and protection form apoptosis (Benvenuti and Comoglio 2007; Corso et al.

2005; Ma et al. 2003; Stuart et al. 2000). To determine if HGF is able to protect BMM from apoptosis, LPS-activated cells were challenged with increasing concentrations of camptothecin. One hour pre-treatment with HGF before the addition of camptothecin had no significant influence on LPS-activated BMM viability (Fig. 7E, first columns). Comparable results were obtained with staurosporine (data not shown). Importantly however, we noted an approximately 30% increase in metabolic activity in control BMM treated with HGF but not challenged with camptothecin suggesting that HGF may induce proliferation (Fig. 7E). Indeed ^{3}H-thymidine incorporation assays showed significantly increased proliferation of LPS-activated BMM upon HGF treatment (269%±10, p=0.001) (Fig. 7F) whereas the proliferation of quiescent BMM was unaffected by HGF (not shown). In summary, these data indicate that, *in vitro*, HGF has no effect on classic macrophage functions such as cytokine and NO secretion, phagocytic activity, and antigen presentation. Rather, HGF enhances the proliferation of LPS-activated macrophages without preventing apoptotic death.

DISCUSSION

HGF and c-met expression in EAE

In this report we show that in EAE at peak disease both HGF and its receptor c-met are expressed in the spinal cord and that c-met is activated. The elevated HGF levels in the CNS in acute EAE correspond well with the elevated levels of HGF found in the CSF of patients with multiple sclerosis in the relapsing acute phase (Tsuboi et al. 2002). In general, the activation of c-met correlated well with HGF expression, except that in some animals at the onset of disease, c-met was activated although HGF levels were still comparable to those in naïve animals. This apparent contradiction may be explained by, for example, the cellular location of the receptor; initial intracellular c-met may be transported to the plasma membrane at the onset of EAE where it would then be available for activation by HGF. Alternatively, natural occurring c-met antagonist such as NK1 and 2 and NK4-like protein (Chan et al. 1991; Raymond et al. 2006), both splice variants of full length HGF, may inhibit c-met activation in the CNS under systemic conditions. CNS injury may down regulate the levels of these antagonists, allowing c-met activation without a preceding increase in HGF.

We show c-met expression is limited to the peak phase of disease, confined to clusters of infiltrating cells in the CNS and mainly expressed by macrophages. Although both DCs and macrophages have been described to be able to express c-met (Chen et al. 1996; Galimi et al. 2001; Ovali et al. 2000; Rutella et al. 2006), in the CNS the majority of the c-met co-localizes with CD11b and not with CD11c, suggesting that only a small population of DCs express c-met. In addition, our *in vitro* observations indicate that both naïve and LPS-matured BMDCs do not express c-met whereas LPS- and TNFα-activated BMM show a robust c-met expression. Although a

microglial origin of the c-met$^+$/CD11b$^+$ cells in the CNS of EAE diseased animals cannot be excluded at present. However, both cultured primary microglia and the microglial cell line BV2 failed to express c-met in response to stimuli that elicit significant c-met expression in BMM, suggesting a CNS-infiltrating macrophage rather than a microglial origin of the c-met$^+$/CD11b$^+$ cells. Importantly the expression of c-met on CD11b$^+$ splenocytes may indicate that cmet$^+$/CD11b$^+$ cells migrate between the spleen and CNS, a notion supported by the chemotactic properties of HGF for many cell types (Benvenuti and Comoglio 2007; Corso et al. 2005). However, the presence of macrophages in the CNS at the onset phase of EAE, prior to the expression of c-met on splenic macrophages contradicts such a scenario.

Notably, c-met staining co-localized only with one of two OPC markers tested. This apparent discrepancy may be explained by the observation that macrophages in CNS lesions can express NG2 ((Bu et al. 2001; Jones et al. 2002), and unpublished observations). However, expression of c-met by OPCs during EAE seems likely since our *in vitro* data and data from others (Yan and Rivkees 2002) show that c-met can be induced by TNFα + IFNγ in OLI-neu cells, both cytokines present at high concentrations in the CNS at peak disease. Interestingly, TGFβ, a cytokine that can induce differentiation and apoptosis of OPCs *in vitro*, had no effect on c-met expression (McKinnon et al. 1993; Schuster et al. 2002). The expression of c-met by OPCs and the role of HGF/c-met signaling in these cells during EAE will require further study which lies outside the scope of the present report.

Surprisingly of all the different stimuli tested only TNFα and LPS were able to induce c-met expression in macrophages. C-met expression by macrophages is poorly described, however a number of groups have studied c-met expression in human peripheral blood monocytes (PBMCs), which can give rise to tissue macrophages.

These reports establish that monocytes are able to express c-met but the precise conditions required differ significantly. Our data is in good agreement with some studies that show induction of c-met expression in human monocytes by pro-inflammatory mediators such as TNFα + IL-6, TNFα + IFNγ and LPS (Beilmann et al. 2000; Chen et al. 1996; Galimi et al. 2001) but contrasts with the findings of others that report IL-1-, IFNγ- and IL-10-induced up-regulation of c-met mRNA (Galimi et al. 2001; Jiang et al. 2001). Inconsistencies between these studies and the present study are probably due to species (human versus murine) and cell type (peripheral blood monocyte versus bone marrow-derived macrophage) differences. However, in general, pro-inflammatory stimuli appear to induce c-met expression in monocytes and macrophages.

Recent research efforts have led to the division of polarized macrophages into different classes based upon the polarizing stimulus and the resulting functional capacity (Martinez et al. 2008; Mosser and Edwards 2008). Classically activated or M1 macrophages develop in response to stimulation with IFNγ and TNFα or microbial products such as LPS and are characterized by their enhanced ability to secrete pro-inflammatory cytokines, endocytosis, and ability to kill intracellular pathogens. Alternatively activated macrophages (M2 macrophages) are critical in type II inflammation, resolution of inflammation, and tissue repair. M2 macrophages express high levels of arginase 1 and, depending on subtype, are induced by IL-4 or IL-13, Fcγ receptor + Toll receptor activation, or deactivation by stimuli such as glucocorticoids, IL-10 or TGFβ (M2a, M2b, M2c, respectively).

Our data show that c-met is preferentially expressed by classically activated M1 macrophages since only TNFα and LPS were able to elicit c-met expression whereas IL-4, IL-13 and IL-10 failed to induce c-met despite increased arginase activity, a

hallmark of M2 macrophages (data not shown). Notably, IL-4, IL-13 and IL-10 also failed to down regulate LPS-induced c-met and alternatively activated macrophages still up-regulated c-met expression in response to LPS. By inference, these observations imply that $CD11b^{+}$/c-met^{+} macrophages in the CNS at peak EAE disease classify as M1 cells. Intriguingly, although classical activation of macrophages normally requires IFNγ in combination with LPS or TNFα and IFNγ significantly enhanced the NO production by LPS-activated BMM, it is not required for LPS- or TNFα-induced c-met expression in BMM. In addition, we provide evidence that TNFα and LPS induce c-met expression in macrophages through two independent pathways and that TNFα triggers c-met expression through TNFR1 signaling and not through TNFR2 signaling. LPS- or TNFα-induced c-met expression appears to be remarkably stable and occurs irrespective of the prior polarization "flavor" of the macrophage.

Function of HGF signaling in macrophages

We show that cytokine expression is unaltered by HGF treatment of LPS-activated BMM, although others have reported HGF-induced increases in chemokines, interleukins, growth factors, and NO in human monocytes (Beilmann et al. 2000; Galimi et al. 2001), In addition, both iNOS mRNA expression and NO production were unaltered by HGF. Thus, intriguingly the production of cytokines and NO appears not to be regulated by HGF in macrophages. Furthermore, a role for HGF signaling in antigen presentation or phagocytosis by macrophages seems unlikely because the expression of MHC class II, and the co-stimulatory molecules CD80 and CD86, were unaltered by HGF treatment of LPS-activated macrophages as was their capacity to phagocytose polystyrene beads.

Well-established cellular responses to HGF include enhanced cell growth, invasion/migration and protection from apoptosis. In our experiments, HGF failed to protect cultured macrophages from camptothecin or staurosporine-induced cell death suggesting that HGF cannot prevent apoptosis in this cell type.

We attempted to assess macrophage migration using a modified Boyden chamber trans-well system. Although Mip-1α improved the migration rate of untreated BMM, the migration rate of LPS-pretreated BMM was unaltered by Mip-1α or HGF (data not shown). Notably because LPS-treated BMM are strongly adherent, which may account for the poor migratory response towards Mip-1α, an alternative migration assay that determines the invasiveness of macrophages into collagen or matrigel for example may be more appropriate. Thus a role for HGF in macrophage migration requires further investigation.

However, HGF did elicit a clear increase in proliferation of LPS-activated macrophages whereas quiescent macrophages were unaffected. These observations suggest that HGF promotes the proliferation of activated macrophages within the CNS in acute EAE, a concept that has profound implications. It would be tempting to speculate that inhibition of HGF signaling may ameliorate the peak phase of EAE by reducing the number of activated macrophages in the CNS.

In this respect it is important to note that in EAE, a potential pro-inflammatory role for HGF signaling in macrophages is supported by a number of our observations. Firstly, c-met^{+}/CD11b^{+} macrophages, HGF, and activated c-met are most abundant in the CNS at the peak phase of EAE. Secondly, the pro-inflammatory cytokine TNFα, which induces c-met expression in cultured macrophages, is highly elevated at this disease stage and plays a crucial role in EAE progression (Probert et al. 2000). Thirdly, IL-4, IL-13 and IL-10 all play important roles in the resolution of

inflammation and initiation of tissue repair, but fail to modulate c-met expression on classically activated macrophages. Intriguingly, our data advocates that c-met expression by activated macrophages may be not limited to EAE but could be a more general feature of macrophages during an inflammatory response. It is interesting to note that in many diseases, such as arthritis (Feuerherm et al. 2001) and lupus nephritis (Ferraccioli and Romano 2008), the expression of HGF and/or c-met are likely to coincide with the presence of macrophages at the inflammatory site.

In summary, our data indicate that HGF/c-met signaling in macrophages may play a pro-inflammatory role in EAE and that c-met could serve as an important therapeutic target in MS. In the light of the current interest in HGF/c-met modulating therapies, it is becoming a pressing issue to investigate the role of HGF signaling in macrophages in a broader scope.

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FIGURE LEGENDS

Figure 1. HGF and the HGF receptor c-met are expressed at peak and remission of EAE in the spinal cord. (A) Analysis of HGF mRNA expression in spinal cord at different EAE stages by quantitative RT-PCR. HGF mRNA expression was significantly increased at peak and remission. Bars represent mean ± SEM of 3 animals per time point. (B and C) HGF protein levels were measured with ELISA in spinal cord lysates (B) and serum (C). HGF levels were significantly elevated in spinal cord at peak and remission and in serum at peak disease. Bars represent mean ± SEM of 4 to 5 animals per time point. (D) Analysis of c-met mRNA expression in spinal cord by quantitative RT-PCR. C-met expression is significantly increased at peak and remission. Bars represent mean ± SEM of 3 animals per time point. (E) C-met immunoprecipitation from spinal cord lysates followed by western blotting for phosphorylated c-met (top panel) and total c-met (lower panel). Phosphorylation of $Tyr^{1230/1234/1235}$ (activation) of c-met is increased at onset, peak and remission. C-met protein levels are elevated at the same EAE stages. Shown is a representative experiment of 4. (F and G) Densitometric analysis of the experiments described in E. Compared to naïve mice, c-met protein is significantly increased in amount (F) and phosphorylation (G) at peak and remission. The highest c-met protein amounts are found at remission and c-met phosphorylation is highest at peak. The quantification of c-met phosphorylation is normalized to total amount of c-met immunoprecipitated. Results are expressed as the mean ± SEM of 4 animals per time point. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus naïve control or as indicated by brackets; Students T-test.

Figure 2. C-met is predominantly expressed by macrophages in the spinal cord at peak EAE. (A-E) Immunohistochemistry for c-met and cellular markers in spinal cord. At peak EAE, c-met (second column) is confined to areas with high numbers of infiltrating cells, easily identified in the DAPI staining (third column) as clustered nuclei. First column shows the expression of the cellular marker indicted and the fourth column shows the merged images of the preceding columns. Last column shows an electronically 5x magnified image of the boxed area in the third column. (A) C-met co-localizes with the OPC marker NG2 (arrows). Many c-met positive cells are NG2 negative (arrowheads). (B) C-met does not co-localize with PDGFR (star), an alternative OPC marker. (C) Many $CD11b^+$ macrophages are c-met positive (arrows). (D) C-met co-localisation with $CD11c^+$ dendritic cells is less frequently observed. (E) $CD4^+$ T cells do not co-localise with c-met. (F) In the remission phase, c-met expression is no longer observed (second column). (A-F) Arrows indicate co-localization of the indicated cellular marker with c-met. Arrowheads depict c-met positive cells that do not co-localize with that marker. Scale bars in the first 4 columns represent 200 μm and 50 μm in the last column.

Figure 3. C-met is expressed by $CD11b^+$ splenocytes at the peak phase of EAE. Flow cytometric analysis of c-met expression by splenocytes isolated at different disease stages. (A) C-met expression is detected at peak EAE on a small population of splenocytes (gated for total live splenocytes). (B) Splenocytes isolated at peak EAE were co-stained with antibodies against c-met, CD11b, CD4 and CD8. Approximately 43% of $CD11b^+$ splenocytes at peak EAE express c-met (third panel). $CD4^+$ and $CD8^+$ splenocytes are c-met negative (first and second dot plot respectively). Fourth dot plot shows isotype control for anti-c-met antibody. (C) Gating on $CD11b^+$ cells

shows that c-met expression is limited to the peak phase of EAE. (A-C) Grey area: isotype control. Results shown are from a representative experiment of 2 EAE series with 3 mice each per time point.

Figure 4. C-met expression can be induced in OPCs and macrophages but not in microglia and dendritic cells. Flow cytometric analysis of c-met expression by different cell types. (A) C-met is not expressed by untreated BV2 cells and cannot be induced by treatment with LPS. (B) C-met expression cannot be detected on untreated, LPS- or TNFα-treated primary microglia. (C) Untreated and LPS-treated BMDCs cells do not express c-met. (D) Treatment of OLI-neu cells with TNFα + IFNγ induces c-met expression. Untreated and TGFβ-treated OLI-neu cells are c-met negative. (E) PECs express c-met on a sub-population of cells. Gating for c-met$^+$ cells shows that these cells are all CD11b and F4/80 positive. (F) Treatment with TNFα or LPS induces c-met expression in BMM whereas untreated BMM do not express c-met. (A-F) Grey area: isotype control. Thin line: c-met expression of untreated cells. Thick line: c-met expression after the indicated stimulus. TNFα 25 ng/ml, IFNγ 100 U/ml, LPS 100 ng/ml, TGFβ 5 ng/ml (40h).

Figure 5. Activation of macrophages by LPS or TNFα induces c-met, in a time- and dose-dependent manner, which can be activated by exogenous HGF. (A) C-met immunoprecipitation (IP) from cultured BMM followed by western blotting for c-met. C-met protein is expressed after macrophage activation with LPS or TNFα (16h) but is absent in untreated macrophages. No Ab: beads-only IP control. No lysate: IP without BMM lysate. (B) C-met immunoprecipitation from LPS-activated BMM followed by western blotting for phosphorylated c-met (top panel) and total c-met

(lower panel). C-met protein is activated (phosphorylated at $Tyr^{1230/1234/1235}$) in BMM in the presence of HGF (50 ng/ml, 1h). (C) LPS- and TNFα dose dependent c-met protein expression in BMM analysed by flow cytometry. Treatment with 10 ng/ml LPS or 25 ng/ml TNFα (40h) is sufficient to induce maximum c-met levels. (D) Time course of LPS- and TNFα-induced c-met protein expression in BMM analysed by flow cytometry. Maximum c-met levels are obtained after 40h of treatment with either 100 ng/ml LPS or 25 ng/ml TNFα. (C-D) Grey area: isotype control. (E) Quantitative RT-PCR analysis for TNFα and c-met of a time-course of LPS treatment of BMM. LPS-induced TNFα mRNA expression (grey bars) precedes c-met mRNA expression (black bars). (F) C-met immunoprecipitation (IP) from wt, TNFR1ko, TNFR2ko, and TNFαko BMM cultures followed by western blotting for c-met. TNFα and LPS induce c-met protein expression in wt, TNFαko and TNFR2ko BMM, but only LPS induces c-met protein expression in TNFR1ko BMM. BMM were treated with LPS or TNFα for 40h. No Ab: beads-only IP control. No lysate: IP without BMM lysate. (A-F) Data are from representative experiments. Bars represent mean ± SEM.

Figure 6. Alternative activation of macrophages does not induce c-met expression and does not prevent or reverse LPS-induced c-met expression. Flow cytometric analysis of BMM treated with various cytokines and combinations thereof. (A) C-met expression can only be induced by LPS and TNFα but not with any of the other stimuli. Grey area: isotype control. Thin line: c-met expression of untreated cells. Thick line: c-met expression after 16h treatment with the indicated stimulus. (B) LPS-induced c-met on BMM is not altered by subsequent treatment with IL-10 or IL-13 but is down regulated by its ligand HGF. Thin line: c-met expression of untreated cells. Thick line: c-met expression after 16h LPS treatment. Grey line: c-met

expression after 16h of treatment with LPS followed by removal, wash and 16h treatment with the indicated stimulus. (C) Alternative activation of BMM with IL-4, IL-10 or IL-13 does not induce c-met expression and does not prevent LPS-induced expression of c-met in BMM. Thin line: c-met expression of untreated cells. Grey line: c-met expression after 16h treatment with the indicated stimulus. Thick line: c-met expression after 16h of treatment with the indicated cytokine followed by removal, wash and 16h treatment with LPS. Shown are histograms representative of at least 3 experiments for each stimulus. Concentrations used are indicated in Table 1.

Figure 7. HGF enhances macrophage proliferation but does not modulate classic macrophage functions. (A) Quantitative RT-PCR for various cytokines, iNOS and c-met in BMM after treatment with LPS and/or HGF. HGF does not modulate cytokine mRNA expression in LPS-treated BMM. White bars: 16h LPS. Grey bars: 16h LPS followed by removal, wash and 16h HGF. Black bars: 16h LPS + HGF followed by removal, wash and 16h HGF. Data are from 5 independent experiments. Bars represent mean ± SEM. (B) Griess test measuring nitrite concentration in medium from LPS-treated BMM cultures. HGF treatment does not alter nitrite production by BMM. Black bars: 16h LPS followed by removal, wash and 16h HGF or no treatment. Grey bars: 40h LPS with or without HGF. Striped bars: 40h LPS + IFNγ with or without HGF. Data are from 3 independent experiments. Bars represent mean ± SEM. (C) Flow cytometric analysis of LPS-treated BMM for different macrophage markers, co-stimulatory molecules and MHC-II. 16h HGF treatment of LPS-treated BMM (lower panels) does not alter expression levels of the indicated markers compared to LPS-only treated BMM (upper panels). One representative experiment of 3 with similar results is shown. (D) BMM phagocytosis of PE-labelled polystyrene

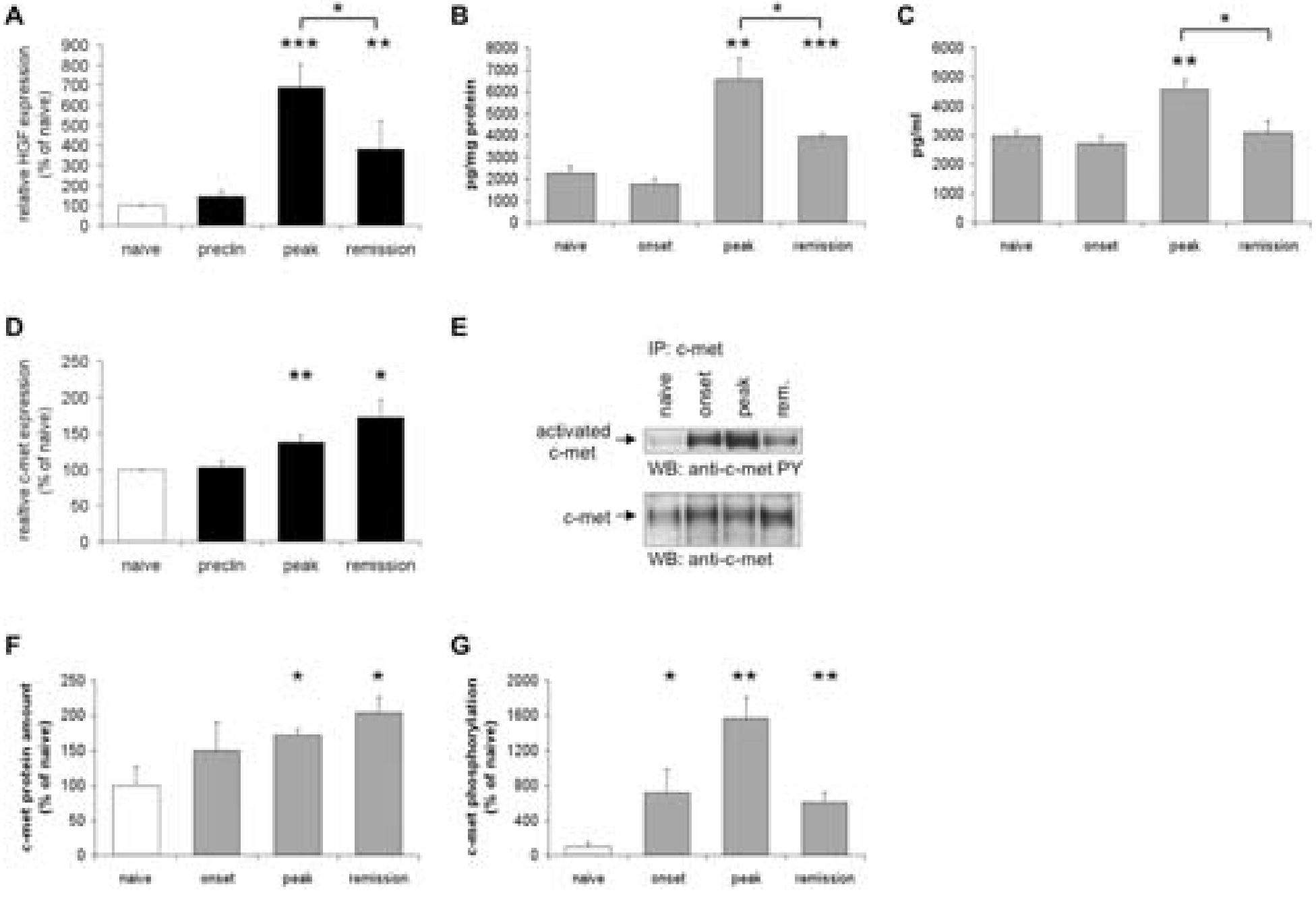
beads. BMM were either left untreated (thin line), treated with LPS for 40h (thick line) or treated with LPS for 40h with addition of HGF in the last hour (grey line) prior to addition of PE-labelled beads for 30 minutes. One representative experiment of 3 with similar results is shown. (E) Survival of camptothecin-treated LPS-activated BMM was determined using a MTT assay. LPS-activated BMM are susceptible to camptothecin-induced (16h) apoptotic death in a dose-dependent manner (black bars). 1h pre-treatment with HGF prior to addition of camptothecin in the continued presence of HGF does not prevent apoptosis (grey bars). One representative experiment with triplicate cultures of 2 experiments with similar results is shown. (F) Proliferation of LPS-treated BMM was assessed by ^{3}H-thymidine incorporation (16h). Continuous presence of HGF during the labelling period significantly enhanced proliferation of LPS-activated BMM. Data are from 3 independent experiments. Bars represent mean ± SEM. ***P<0.001 versus control; Students T-test.

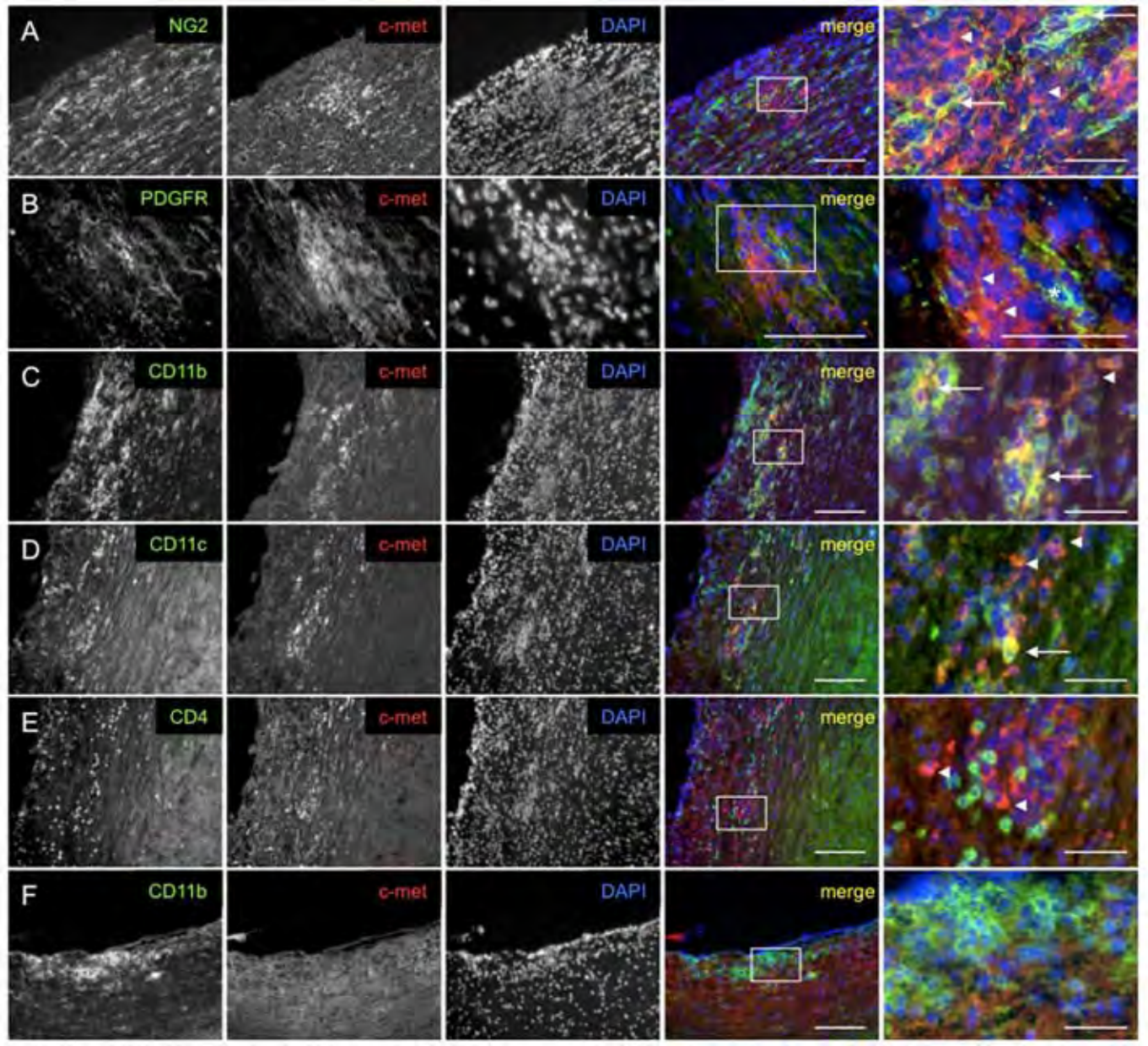
Table 1. Cytokines tested for c-met induction on BMM.

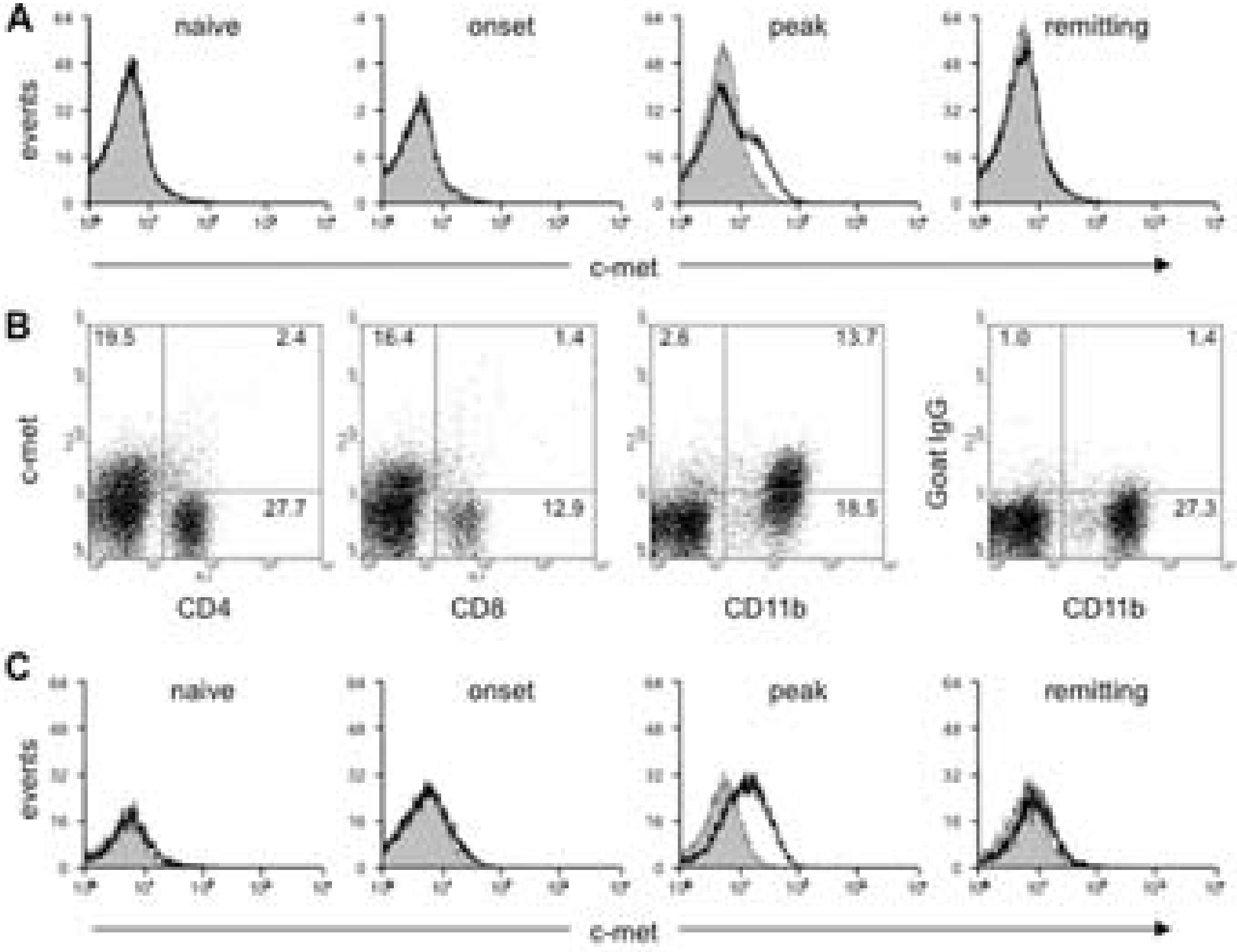
Stimulus	Concentration	c-met regulation?
IL-1β	10, 30* ng/ml	No
IL-2	20*, 100 ng/ml	No
IL-3	20 ng/ml	No
IL-4	10* U/ml	No
IL-6	20* ng/ml	No
IL-8	20* ng/ml	No
IL-10	10, 20, 30* ng/ml	No
IL-12	100* ng/ml	No
IL-13	10, 30* ng/ml	No
IL-17	100* ng/ml	No
Mip-1α	75, 150, 225 ng/ml	No
RANTES	50, 100, 150 ng/ml	No
TGFβ1	0.2, 2, 20 ng/ml	No
TGFβ2	0.2, 2, 20 ng/ml	No
TGFβ3	0.2, 2, 20 ng/ml	No
IFNγ	5, 10, 20*, 50, 100 ng/ml	No
TNFα	5, 10*, 20, 50, 100 ng/ml	**Up**
TNFβ	10 ng/ml	No
HGF	2.5, 5, 10, 20, 40, 50*, 75, 80, 100 ng/ml	**Down**
L-NMA	100, 250 μM	No
PMA	10 ng/ml	No
LPS (B5 and B8)	0.1, 1, 10, 100*, 1000 2500 ng/ml	**Up**

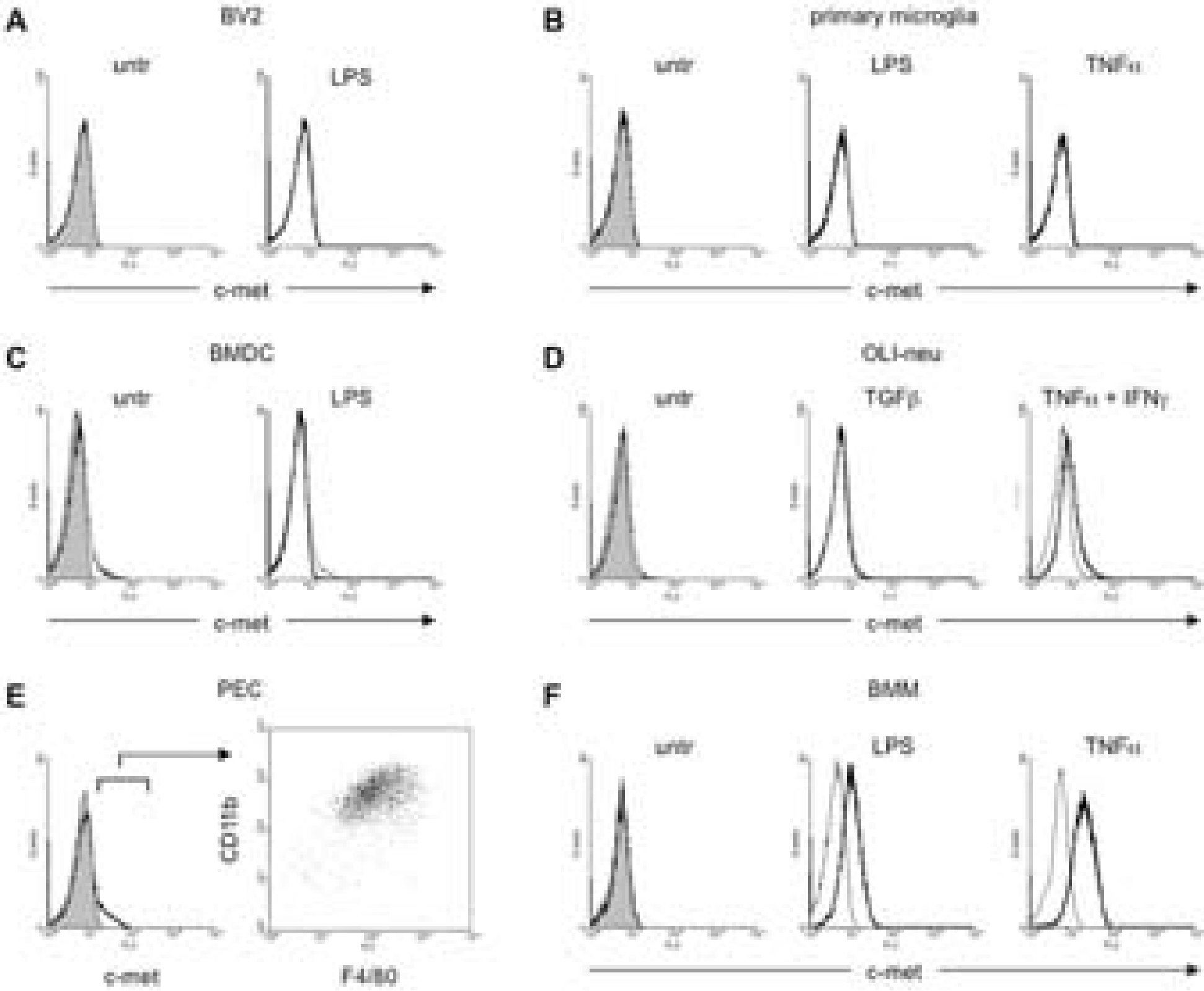
Treatment for 40h, analyzed by flow cytometry

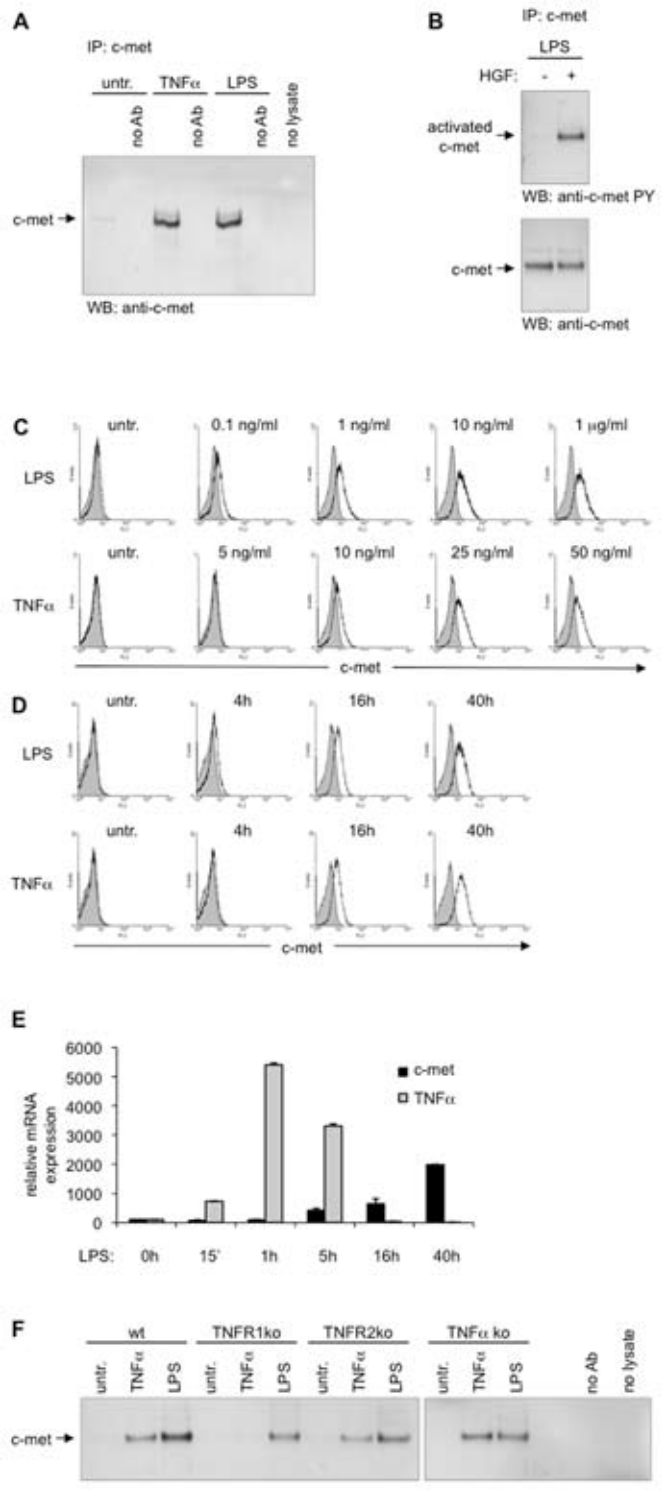
* Concentration used in Fig. 6

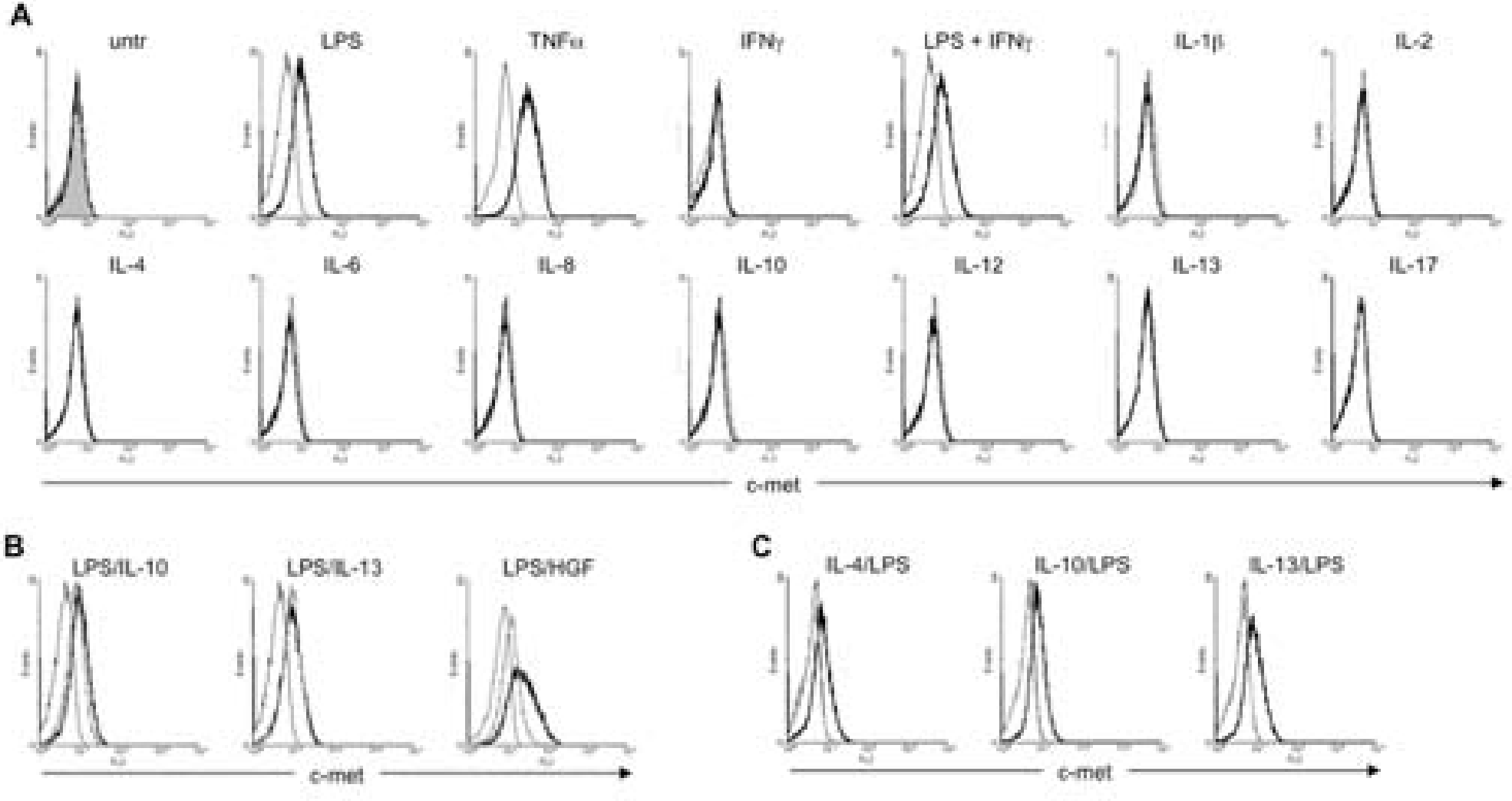

A
relative HGF expression (% of naive)
naive
precln
peak
remission
B
pg/mg protein
naive
onset
peak
remission
C
pg/ml
naive
onset
peak
remission
D
relative c-met expression (% of naive)
naive
precln
peak
remission
E
IP: c-met
naive
onset
peak
rem.
activated c-met
WB: anti-c-met PY
c-met
WB: anti-c-met
F
c-met protein amount (% of naive)
naive
onset
peak
remission
G
c-met phosphorylation (% of naive)
naive
onset
peak
remission

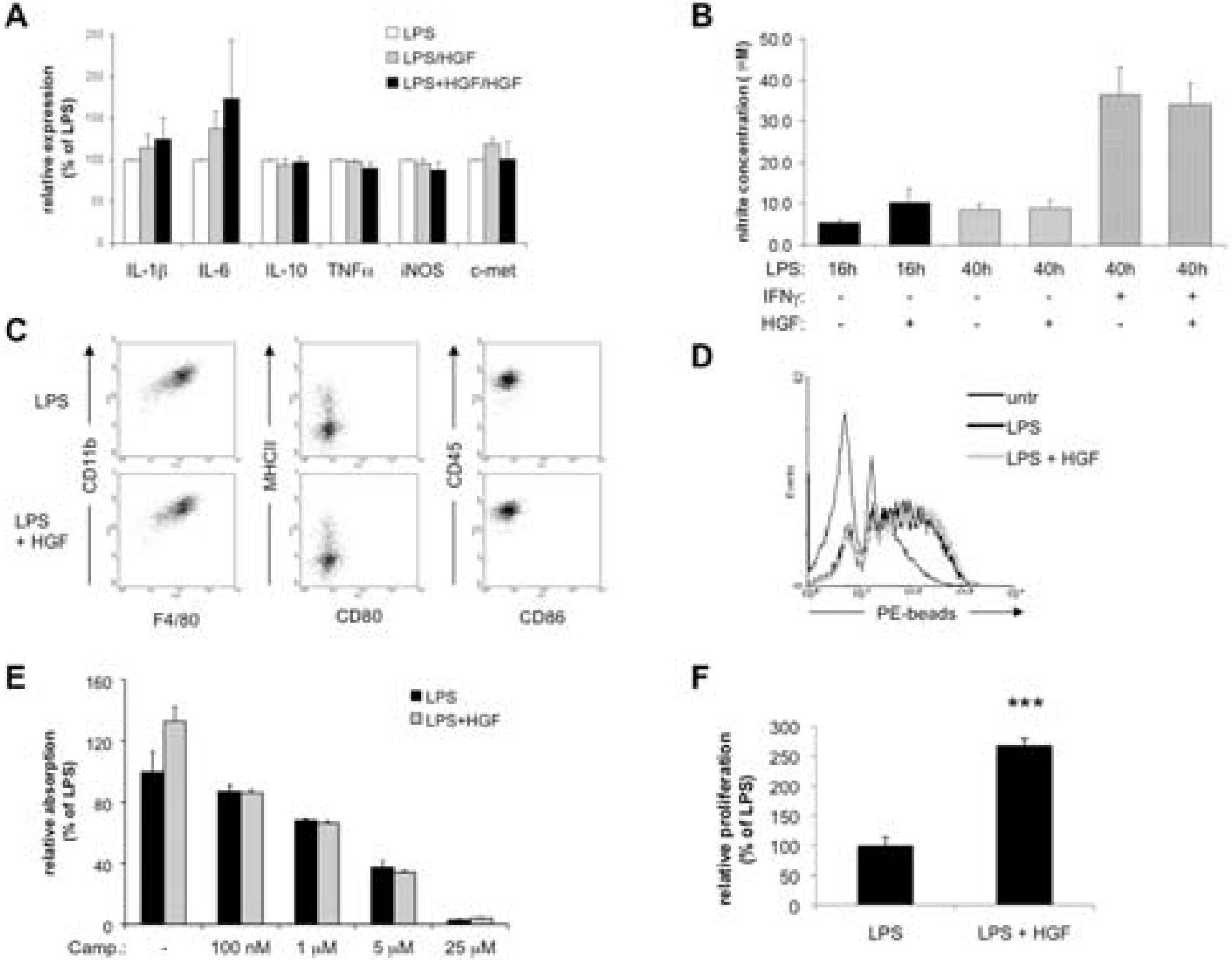

A
NG2
c-met
DAPI
merge
B
PDGFR
c-met
DAPI
merge
C
CD11b
c-met
DAPI
merge
D
CD11c
c-met
DAPI
merge
E
CD4
c-met
DAPI
merge
F
CD11b
c-met
DAPI
merge


A
naive
onset
peak
remitting
events
c-met
B
c-met
19.5
2.4
27.7
16.4
1.4
12.9
2.8
13.7
18.5
1.0
1.4
27.3
Goat IgG
CD4
CD8
CD11b
CD11b
C
naive
onset
peak
remitting
events
c-met


A
BV2
untr
LPS
c-met
B
primary microglia
untr
LPS
TNFα
c-met
C
BMDC
untr
LPS
c-met
D
OLI-neu
untr
TGFβ
TNFα + IFNγ
c-met
E
PEC
CD11b
c-met
F4/80
F
BMM
untr
LPS
TNFα
c-met


A
IP: c-met
untr.
TNFα
LPS
no Ab
no Ab
no Ab
no lysate
c-met
WB: anti-c-met
B
IP: c-met
LPS
HGF: - +
activated c-met
WB: anti-c-met PY
c-met
WB: anti-c-met
C
LPS
untr.
0.1 ng/ml
1 ng/ml
10 ng/ml
1 μg/ml
TNFα
untr.
5 ng/ml
10 ng/ml
25 ng/ml
50 ng/ml
c-met
D
LPS
untr.
4h
16h
40h
TNFα
untr.
4h
16h
40h
c-met
E
relative mRNA expression
6000
5000
4000
3000
2000
1000
0
c-met
TNFα
LPS: 0h 15' 1h 5h 16h 40h
F
wt
TNFR1ko
TNFR2ko
TNFα ko
untr.
TNFα
LPS
no Ab
no lysate
c-met


A
untr
LPS
TNFα
IFNγ
LPS + IFNγ
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
IL-12
IL-13
IL-17
c-met
B
LPS/IL-10
LPS/IL-13
LPS/HGF
c-met
C
IL-4/LPS
IL-10/LPS
IL-13/LPS
c-met


A
LPS
LPS/HGF
LPS+HGF/HGF
relative expression (% of LPS)
IL-1β
IL-6
IL-10
TNFα
iNOS
c-met
B
nitrite concentration (µM)
50.0
40.0
30.0
20.0
10.0
0.0
LPS: 16h 16h 40h 40h 40h 40h
IFNγ: - - - - + +
HGF: - + - + - +
C
LPS
LPS + HGF
CD11b
MHCII
CD45
F4/80
CD80
CD86
D
untr
LPS
LPS + HGF
PE-beads
E
LPS
LPS+HGF
relative absorption (% of LPS)
160
120
80
40
0
Comp.: - 100 nM 1 µM 5 µM 25 µM
F
***
relative proliferation (% of LPS)
300
250
200
150
100
50
0
LPS
LPS + HGF