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Microglia activation by SIV-infected macrophages: alterations in morphology and cytokine secretion

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Abstract HIV infection in the brain and the resultant encephalitis affect approximately one third of individuals infected with HIV, regardless of treatment with antiretroviral drugs. Microglia are the resident phagocytic cell type in the brain, serving as a "first responder" to neuroinvasion by pathogens. The early events of the microglial response to productively infected monocyte/macrophages entering the brain can best be investigated using in vitro techniques. We hypothesized that activation of microglia would be specific to the presence of simian immunodeficiency virus (SIV)-infected macrophages as opposed to responses to macrophages in general. Purified microglia were grown and stimulated with control or SIV-infected macrophages. After 6 h, aliquots of the supernatant were analyzed for 23 cytokines using Millipore nonhuman primate-specific kit. In parallel experiments, morphologic changes and cytokine expression by individual microglia were examined by immunofluorescence. Surprisingly, the presence of macrophages was more important to the microglial response rather than whether the macrophages were infected with SIV. None of the cytokines examined were unique to co-incubation with SIV-infected macrophages compared with control macrophages, or their supernatants. Media from SIV-infected macrophages, however, did induce secretion of higher levels of IL-6 and IL-8 than the other treatments. As resident macrophages in the brain, microglia would be expected to have a strong response to infiltrate innate immune cells such as monocyte/macrophages. This response is triggered by incubation with macrophages, irrespective of whether or not they are infected with SIV, indicating a rapid, generalized immune response when infiltrating macrophages entering the brain.

Keywords Cytokine · Chemokine · Microglia · HIV · Neuroinflammation · Neuropathogenesis

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Introduction

Approximately one third of HIV-infected individuals develop HIV-associated neurocognitive disorders (Kraft-Terry et al. 2009). HIV, and the ancestral virus (simian immunodeficiency virus, SIV), enters the brain during routine immune surveillance (Williams and Hickey 1995) within monocyte-derived macrophages, a so-called Trojan horse (Narayan et al. 1982). Additional monocytes are recruited to this initial site of infection, where they can also become infected (Williams et al. 2001).

The environment within the parenchyma is sampled by ramified microglia (Roberts et al. 2004), which are capable of facilitating a targeted immune response (McKimmie and Fazakerley 2005). There is a change in the phenotype of microglia, from ramified to amoeboid morphology within 4 h (Skibo et al. 2000). Activated microglia secrete pro-inflammatory

cytokines and chemokines (Renner et al. 2011a, b; Williams et al. 2009; Bernardino et al. 2008), selectively recruiting leukocytes to the site of infection. In SIV/HIV infection it is not known that the microglial response is a general response to macrophages or is specific to SIV-infected macrophages.

Previous studies have shown SIV-infected microglia secrete increased cytokines (Sopper et al. 1996). However, infiltrating monocyte-derived macrophages would also be expected to induce pro-inflammatory signaling cascades. We hypothesized that SIV-infected macrophages would induce secretion of a specific "barcode" of cytokines and chemokines in purified microglia (McKimmie and Graham 2010). These studies were therefore untaken to differentiate a pathogen-specific, from generalized macrophage ingress into the brain.

Morphology of microglia was observed following co-incubation with SIV-infected or control macrophages and their supernatants. Additionally, we monitored the secretion of cytokines and chemokines previously shown to be important in AIDS neuropathogenesis (Orandle et al. 2001, 2002). Cytokines and chemokines that were found to be differentially regulated by SIV in microglia were examined at the individual cellular level by immunofluorescent microscopy.

Cultures of microglia were shown to have increased chemokine (C-C motif) ligand 2 (CCL2), granulocyte macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor α (TNF-α) when cultured with either control or SIV-infected bone marrow-derived macrophages, and increased levels of interleukin (IL)-6, IL-8, and vascular endothelial growth factor (VEGF) in the presence of SIV-infected macrophage supernatants. These data suggest that microglia rapidly respond to both direct interaction with macrophages, and to factors secreted by SIV-infected macrophages.

Materials and methods

Morphological examination of microglia in situ

A complete necropsy of SIV-infected macaques at the Tulane National Primate Research Center includes collection of a complete set of brain tissues including multiple blocks of cerebral cortex, subcortical gray and white matter, thalamus, basal ganglia, cerebellum, and spinal cord.

Golgi staining

Tissues were stained using the Golgi–Cox procedure developed by Gibb and Kolb (1998), with the following modifications: tissue blocks (approximately 5 mm) were fixed in paraformaldehyde, and immersed in Golgi–Cox solution for 2 weeks, followed by immersion in sucrose solutions as described. Sections were then cryostat sectioned (100 μm), and mounted onto gelatin-coated slides. Golgi–Cox stain was developed as per the published method.

Immunofluorescent staining

Formalin-fixed, paraffin-embedded tissues were sectioned at 6 μm and mounted onto positively charged glass slides. Sections were baked for 1 h at 60°C, deparaffinized in xylene, and then rehydrated in graded concentrations of ethanol. Antigen retrieval was carried out for 20 min using a microwave on high power and a citrate-based antigen unmasking solution (Vector Labs, Burlingame, CA). Tissues were blocked in a 10 % normal goat serum solution (GIBCO/Invitrogen, Carlsbad, CA) for 1 h at room temperature before AIF-1 antibody (1:50 dilution, Wako, Taiwan) was applied overnight at 4°C, washed three times with PBS with 0.2 % fish skin gelatin (PBS/FSG), and then incubated in the dark for 60 min at room temperature with secondary antibodies directly conjugated with Alexa 488 (green; Molecular Probes/Invitrogen, Carlsbad, CA). Sections were washed three times in PBS/FSG, and cover slipped with anti-quenching reagent (Molecular Probes/Invitrogen).

Culture of microglia

Mixed glial cultures were prepared as previously described (Williams et al. 2001). In brief, frontal cortices were aseptically obtained from juvenile normal Indian origin rhesus macaques at necropsy. Meninges were removed, the tissue diced and incubated with trypsin (0.25 %, Invitrogen, Carlsbad, CA) and DNAse (4 U/ml, Sigma, St. Louis, MO) for 1 h at 37°C. The resulting slurry was triturated and passed through a 110-μm pore filter and centrifuged at 1,000 rpm for 5 min. Pellets were washed twice and resuspended in M199 (Mediatech, Manassas, VA) supplemented with 10 % fetal calf serum (FCS), 10 mM glucose, and 4 ml of 7.5 % sodium bicarbonate (Invitrogen). As cultures approached confluence, microglia were removed from cultures by vigorous shaking. These microglia were then plated at high density and cultured in the presence of GM-CSF (0.5 ng/ml; Peprotech, Rocky Hill, NJ). Microglia cultures were used 4 days after plating.

Culture and infection of macrophages

Bone marrow-derived macrophages (BMDM) were cultured as previously described (Ivey et al. 2009). Bone marrow was obtained from juvenile normal macaques aseptically at necropsy, vortexed, and filtered through 70-μm pore filters. Macrophages were grown in IMDM media (Mediatech) supplemented with 10 % FCS and antibiotics (Invitrogen). BMDM were infected by incubating with SIVmac251 (a neuropathogenic strain) using 100 $TCID_{50}$ per T25 flask for 4 h before washing and culturing for a further 48 h. At that point, media were harvested, pooled, and macrophages

trypsinized. The macrophages were resuspended in fresh macrophage media.

Stimulation of glial cultures

To model the early events following SIV neuroinvasion, purified microglia were washed twice with PBS and incubated for 6 h with one of the following: control BMDM supernatant (CMS), SIV-infected BMDM supernatant (SIMS), control BMDM cells, or SIV-infected BMDM cells.

Quantification of cytokines secreted

Samples were taken immediately after addition of macrophages or conditioned media and again after 6 h. Aliquots were frozen at −80°C until cytokines were measured by multiplex. Each aliquot underwent a single freeze-thaw cycle. Quantification of secreted cytokines was performed with the Luminex platform and Milliplex nonhuman primate-specific kits (Millipore, Billerica, MA) using the manufacturer's instructions. Each sample was measured in duplicate and averages plotted. For samples stimulated with conditioned media, the initial levels were subtracted from those measured 6 h later. Thus, we are only reporting de novo secretion of cytokine by microglia.

Exclusion criteria

Each analyte in the kit is theoretically quantifiable from 1 through 10,000 ng/ml. De facto ranges are smaller and vary by analyte. For our analyses, any analyte where greater than 50 % of the values were extrapolated beyond the range of the working standard curve was excluded. Analytes that were excluded as a result of this are: IFN-γ, IL-1ra, CCL4, CD40L, TGFβ, and IL-18. Some analytes returned measured values at or near zero for each sample: it is known, for example, that IL-10 was not cross-reactive with rhesus macaque proteins. It was out of the scope of these experiments to determine if other near zero values were accurate measurements, or the result of poor cross-reactivity. Analytes that were excluded for this reason are: IL-1b, IL-4, IL-5, IL-10, IL-12, IL-13, IL-15, IL-17, and CCL3.

Statistical analyses

To determine significant changes in cytokine levels, ANOVA with Tukey's post test was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, California).

Blocking studies and image analyses

Parallel studies were performed using purified microglia cultured on chamber slides. As with the secretion studies, microglia were cultured for 4 days before washing twice with PBS and the addition of macrophages or their supernatants. In the final 2 h of incubation, secretion of cytokines was blocked with Brefeldin A (5 μg/ml, Renner et al. 2011a). Cultures were fixed with 2 % paraformaldehyde in PBS for 20 min before staining for immunofluorescent microscopy. Antibodies used were as follows: GFAP (Sigma; clone GA5, 1:1,000 dilution), IL-6 (Chemicon; MAB1033, 1:2,000 dilution), CCL2 (Pharmingen; 5D3-47, 1:500 dilution), TNF-α (Santa Cruz; goat, 1:250 dilution), and VEGF (Santa Cruz; rabbit, 1:250 dilution). Cells were permeabilized with 0.1 % Triton (in PBS with 1 % BSA) and stained for relevant cytokine. Antibodies were detected with secondary antibodies directly conjugated with Alexa 488 or 568 (Molecular Probes/Invitrogen).

Results and discussion

These studies were undertaken to investigate acute microglial activation in the context of HIV neuroinvasion. The presence of inflammatory macrophages is one of the hallmarks of HIV infection of brain. Therefore we sought to determine if the lentiviral infection status of BMDM was a determinant in the activation status of microglia or if the secreted factors of infected macrophages were more important. As these studies used microglia in culture, we began by comparing the morphology of microglia in situ and in vitro.

Morphological examination of microglia in situ

Microglia alter their morphology from a ramified morphology (Fig. 1a) to amoeboid (Fig. 1b) in neuroinflammation. AIF-1, an actin-binding protein, is considered to be a microglial-specific marker within the normal brain (Sopper et al. 1996; Kohler 2007; Ohsawa et al. 2004). As such, AIF-1 is ideally suited to examining morphological changes in microglia. Cells expressing AIF-1 in the brain were ramified (C), changing to amoeboid—and considerably smaller—on activation (D).

Ramified microglia sample their environment using long processes. These processes retract on activation, allowing the microglia to migrate to the source of infection (Tassi et al. 2006). We were interested to determine if SIV-infected macrophages would activate cultured microglia to alter from a ramified to amoeboid morphology.

Morphological examination of microglia in vitro

To confirm the microglial cells were pure, cultures were checked for contaminating astrocytes. No GFAP immunopositive cells were observed in the microglial cultures (25 nonoverlapping fields, not shown). When stained with AIF-1 antibody, cultured microglia have a ramified morphology

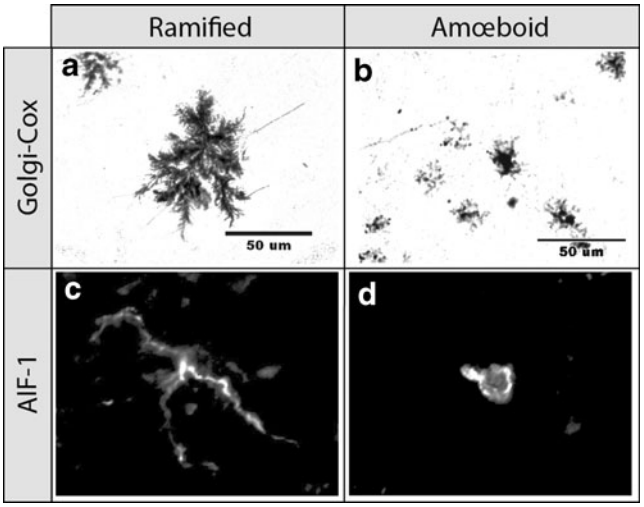

Fig. 1 Morphological characterization of microglia in situ. Typical morphology of a ramified microglia in nonhuman primate cortex stained by the Golgi–Cox method (**a**). In brains with SIV encephalitis, microglia take on an amoeboid, activated phenotype (**b**). This is mirrored using immunofluorescent staining for AIF-1 with ramified morphology (**c**) becoming amoeboid in areas of inflammation (**d**)

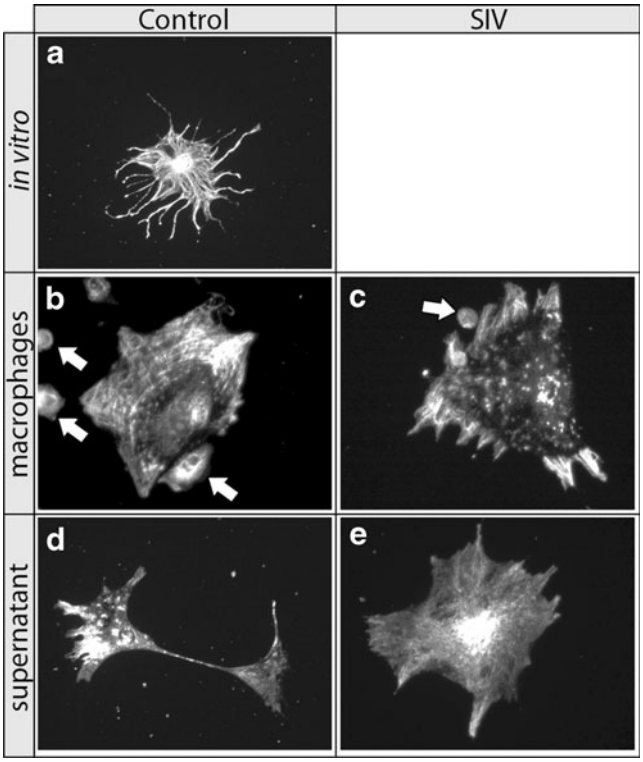

Fig. 2 Morphological characterization of microglia in vitro. Cultured microglia have abundant processes radiating from the body when visualized with AIF-1 immunofluorescent staining (**a**). When incubated with noninfected macrophages (*arrows*), cultured microglia have an activated, amoeboid morphology (**b**), with evident stress fibers. When incubated with SIV-infected macrophages, the AIF-1 is localized to the periphery (**c**), with amoeboid morphology. Incubation with control BMDM supernatant induced an intermediary phenotype (**d**), with some process extension is still present, but not to the extent of ramified microglia. SIV-infected BMDM supernatant induced amoeboid morphology (**e**), with absence of processes, but with AIF-1 staining present throughout the cell

with many fine processes and a central nucleus (Fig. 2a) similar to that observed in the normal brain. When BMDM were co-incubated with cultured microglia for 6 h, the fine processes coalesce, or disappear entirely (Fig. 2b), with orthogonal bands of AIF-1 staining common and intense peripheral staining. There was an apparent morphological difference if the macrophages were infected with pathogenic SIVmac251 (Fig. 2c): the orthogonal bands of AIF-1 expression were not observed, with no discernible staining pattern. AIF-1 expression was not specific to any cellular region. Some cells had perinuclear AIF-1expression, other cells towards the periphery. Microglia could be easily distinguished from co-incubated macrophages, as, in the 6 h incubation, the recently adherent macrophages had yet to spread substantially (arrows).

Incubation of microglia with cell-free macrophage supernatants revealed more subtle changes in morphology. CMS induced a less severe phenotype than did BMDM with approximately half of microglia (7 out of 13) retaining some processes (Fig. 2d). SIMS (Fig. 2e) was noted to induce a more amoeboid phenotype, with few cells retaining any processes (3 out of 14). AIF-1 expression was fairly evenly distributed across the cell with either CMS or SIMS.

Thus, microglia in culture appear to behave in a manner similar to that observed in vivo, with a ramified morphology that is rich in AIF-1 expression. Activation appears to cause these processes to coalesce in a stimulus-dependent manner.

Cytokine secretion in microglial cell cultures

To determine which interleukins and other cytokines/chemokines were differentially regulated in microglia in the context of HIV neuroinflammation, we cultured purified microglia with control or SIV-infected BMDM or their supernatants. For each treatment, the initial level of cytokine was subtracted from final levels. Therefore, we are reporting the de novo secretion of cytokine induced within 6 h by BMDM or cell-free macrophage supernatant.

There was a low level of secretion of IL-2 (Fig. 3a) when macrophages were present, with a larger increase in the presence of control BMDM (29.3 pg/ml) compared to SIV-infected BMDM (19.93 pg/ml). This difference was not, however, statistically significant ($p>0.05$). SIMS was sufficient to induce an increase in IL-2 secretion to a level approaching that of SIV-infected BMDM (14.81 pg/ml). This was higher than that observed for CMS (3.45 pg/ml). The only statistically significant difference was between control macrophages and control media ($p<0.01$). These levels were still low, in agreement with previously published data (Marcondes et al. 2007). SIV encephalitis (SIVE)/HIV encephalitis (HIVE) is characterized by the presence of

inflammatory macrophages, with infrequent T cells (Williams and Hickey 2002). It is therefore not surprising that we saw only a modest increase in a cytokine associated with T cell proliferation.

Substantial quantities of IL-6 secretions were noted in all four treatment groups (Fig. 3b). SIV-infected BMDM (644 pg/ml) induced secretion of IL-6 that was not significantly different from control BMDM (589 pg/ml). The secretion was significantly higher when SIMS was added to the glial cultures compared to CMS ($p<0.001$). CMS induced a secretion of 330 pg/ml. SIMS induced secretion of IL-6 greater than 6,000 pg/ml which exceeded the working standard curve for the analyte. The infectious state of the BMDM made no difference to the quantity of IL-6 secreted ($p>0.05$). A factor secreted by SIV-infected BMDM induced a much higher level of IL-6 than the BMDM themselves. Microglia are therefore capable of rapid secretion of IL-6 in the presence of pro-inflammatory mediators, indicating IL-6 may be synthesized by microglia and stored awaiting a signal to induce the secretion. There are well-characterized neurotoxicity manifestations associated with HIV infection (Williams and Hickey 2002), including increased IL-6 secretion of the neurotoxic IL-6 by the glia in response to gp120 (Shah et al. 2011). Therefore, rapid secretion of high levels of IL-6 by microglia would be anticipated to be a detrimental effect of SIV-infected macrophage infiltration into the brain (Sugama et al. 2009).

IL-8 (Fig. 3c) was secreted at the same level by purified microglia regardless of whether BMDM were infected with SIV (1,095 pg/ml) or uninfected (1,181 pg/ml). CMS induced a significantly lower level of IL-8 (490 pg/ml, $p<0.001$). Thus, contact with macrophages induced a secretion of IL-8 over and above that induced by supernatant alone. SIMS induced secretion of greater than 5,000 pg/ml of IL-8, which exceeded the working standard curve for the analyte. Expression of IL-8 has been recently demonstrated to be increased in microglia in HIVE brain tissue (D'Aversa et al. 2008), possibly in response to gp120 (Shah and Kumar 2010). Our data suggest that the increased IL-8 expression observed in glial nodules by the Berman group may be largely to due to a factor secreted by HIV-infected macrophages. IL-8 has been shown to have neurotoxic effects, and thus plays a role in cognitive dysfunction associated with HIV (Xiong et al. 2003).

Monocyte chemotactic protein 1 (CCL2) was secreted at significantly higher levels in the presence of BMDM than the supernatant (Fig. 3d, $p<0.01$), with SIV-infected BMDM inducing secretion of 3,513 pg/ml and control BMDM

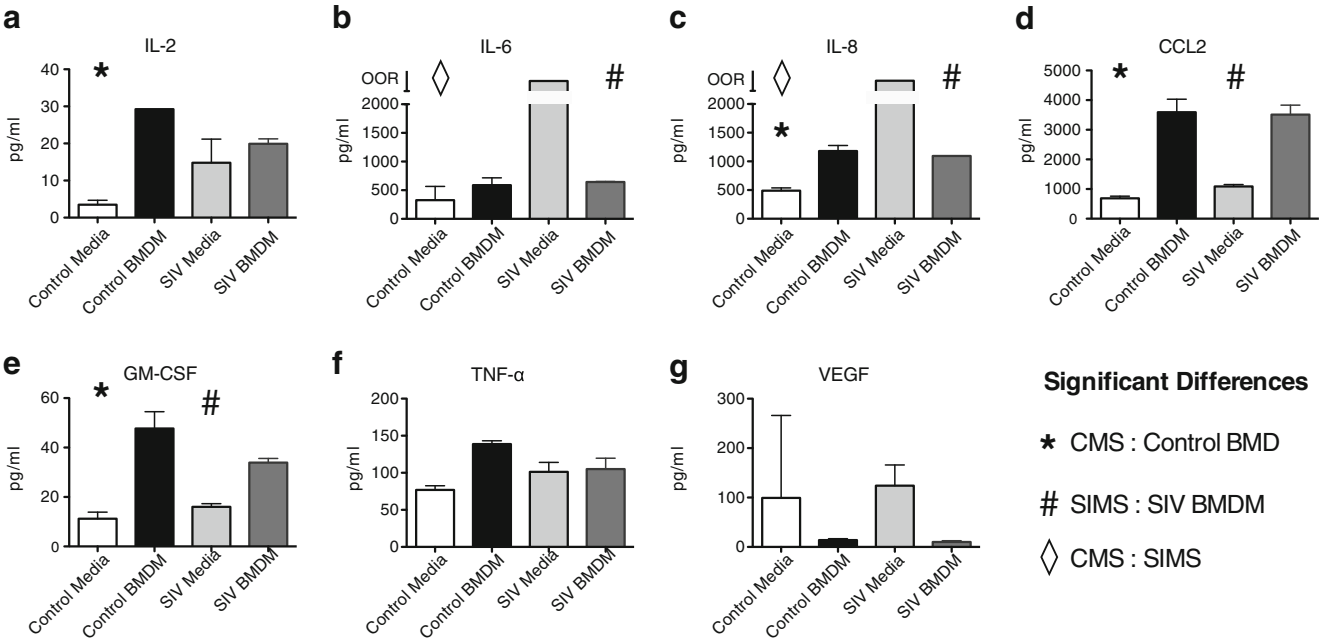

Fig. 3 Cytokine secretion by cultured microglia in the presence of SIV-infected macrophages and their supernatants. Incubation of microglia with control macrophages induced secretion equal or greater than that of SIV macrophages for most cytokines, consistent with the presence of macrophages as the inflammatory stimulus (**a**–**g**). SIV-infected macrophages induced responses similar to the levels induced by control macrophages, often inducing a slightly lower response. SIV-infected BMDM supernatant induced the highest responses of IL-6, IL-8, and VEGF (**b**, **c**, and **g**). Control BMDM supernatant induced the lowest values for most cytokines, with the exception of VEGF (**g**). Statistically significantly different levels of cytokines expression between control BMDM supernatant and control BMDM is represented by *asterisk*; SIV-infected BMDM supernatant and SIV-infected BMDM by *number sign*; control BMDM supernatant and SIV-infected BMDM supernatant by *empty diamond*. There was no significant difference between levels of cytokines secreted by control BMDM and SIV BMDM

inducing 3,593 pg/ml. CCL2 has been reported to be increased in CSF of HIV- (Cinque et al. 1998) and SIV-infected individuals (Mankowski et al. 2004), but not correlated with levels in plasma in either humans (Sevigny et al. 2007) or macaques (Renner et al. 2012). Additionally, CCL2 was not observed in brains of macaques with SIVE (Sasseville et al. 1996). Therefore, the source of CCL2 has been open to debate in the context of HIV neuropathogenesis. However, SIMS did induce a marginally higher level of CCL2 (1,087 pg/ml) than did CMS (687 pg/ml, $p>0.05$), indicating that microglia were secreting significantly higher levels of CCL2 in response to contact with inflammatory macrophages than secreted factors.

Control BMDM induced secretion of 47.72 pg/ml GM-CSF compared with 33.91 pg/ml by SIV-infected BMDM (Fig. 3e, $p>0.05$). This was higher than the levels of GM-CSF induced by media alone: SIMS induced 16.04 pg/ml, and CMS induced 11.23 pg/ml, which were not significantly different from each other ($p>0.05$). Contact with BMDM induced significantly higher levels of GM-CSF compared their respective media ($p<0.05$). It should be noted that the microglia had GM-CSF in the culture medium until washed with PBS immediately prior to experimental treatments. GM-CSF inhibits multinucleate giant cell (MNGC) formation (Tambuyzer and Nouwen 2005). MNGC are a hallmark of late-stage HIV infection in the brain (Bell 1998). It is probable that these late-stage events are not mimicked in our model of acute HIV infection.

TNF-α (Fig. 3f) was found to be secreted at highest levels when microglia were incubated with control BMDM (138 pg/ml) compared with SIV-infected BMDM (105 pg/ml). SIMS induced secretion of 101 pg/ml TNF-α, with CMS 75 pg/ml. This, again, was surprising, especially as we and other groups have shown TNF-α to be upregulated when SIV-infected macrophages are present in brain (Orandle et al. 2002). However, as there was no significant difference between secretion induced by control macrophages and SIV-infected macrophages ($p>0.05$), it is apparent that the presence of macrophages may be just as important as that they are productively infected with SIV for the induction of TNF-α secretion by microglia. It is also possible that microglia are not the primary producers of TNF-α in encephalitis: astrocytes and macrophages are also known to produce TNF-α (El-Hage et al. 2011).

VEGF was secreted (Fig. 3g) following incubation with either SIV-infected BMDM (9.98 pg/ml) or control BMDM (13.92 pg/ml). This was approximately tenfold lower than levels secreted when microglia were incubated with SIMS (124 pg/ml) or CMS (99 pg/ml). This suggests that microglia secrete VEGF in response to a secreted factor from macrophages (whether SIV infected or not). As the levels are low in response to BMDM, it is probable that this factor is secreted slowly, and is upregulated when BMDM are infected with SIV. VEGF secretion could lead to astrogliosis (Mani et al. 2010) and increased permeability of the blood–brain barrier (Suidan et al. 2010).

Our data show a cytokine profile consistent with the hypothesis that, under inflammatory conditions, microglia behave similarly to activated macrophages (Williams and Hickey 2002). Namely, expression of IL-6, IL-8, GM-CSF, and TNF-α all increase in the presence of SIV-infected macrophages. Low levels of IL-2 were consistent with the nature of SIVE/HIVE as a predominately macrophage disease (ibid).

Surprisingly, co-incubation with uninfected BMDM resulted in cytokine secretion comparable to that of SIV-infected BMDM. This suggests that cell-to-cell contact is sufficient to induce an immune response. As perivascular macrophages are generally held within the basal lamina (Abbott et al. 2006), microglia are unlikely to encounter macrophages under normal conditions in the brain, so it is possible that resting microglia respond to any contact with macrophages. Increased macrophages in the brain that have been associated with rapid disease progression (Westmoreland et al. 1998) have also been shown to have an altered cytokine profile in the periphery (Orandle et al. 2001; Rivera-Amill et al. 2010).

Visualization of select cytokines in microglial cultures

It is not possible to determine the cellular source of secreted cytokines with mixed cell cultures using multiplex. The BMDM are just as likely to be the source of cytokines as microglia. In order to address this limitation, we cultured microglia on chamber slides in the presence of BMDM or their supernatants, blocking secretion of cytokines with brefeldin A. BMDM were differentiated from microglia based on morphology. We selected one each of interleukins (IL-6), chemokines (CCL2), angiogenic cytokines (VEGF), and TNF-α as a cytokine previously reported in SIV encephalitis (Orandle et al. 2002).

IL-6 was detected at low levels within microglia in culture (Fig. 4a) or when treated with either control (b), SIV-infected BMDM (c), or CMS (d), consistent with multiplex data (Fig. 3c). There was considerably increased expression of IL-6 observed when microglia were incubated for 6 h with SIMS (e).

Microglia in culture express a low level of CCL2 (Fig. 4f) that is increased following incubation with either control (g) or SIV-infected (h) bone marrow-derived autologous macrophages. Incubation with CMS (i) or SIMS (j) induced a lower level of CCL2 than did BMDM. These data confirm the multiplex array (Fig. 3g).

Control microglia had very low levels of TNF-α (Fig. 4k), with increased expression following incubation with macrophages, regardless of whether the macrophages

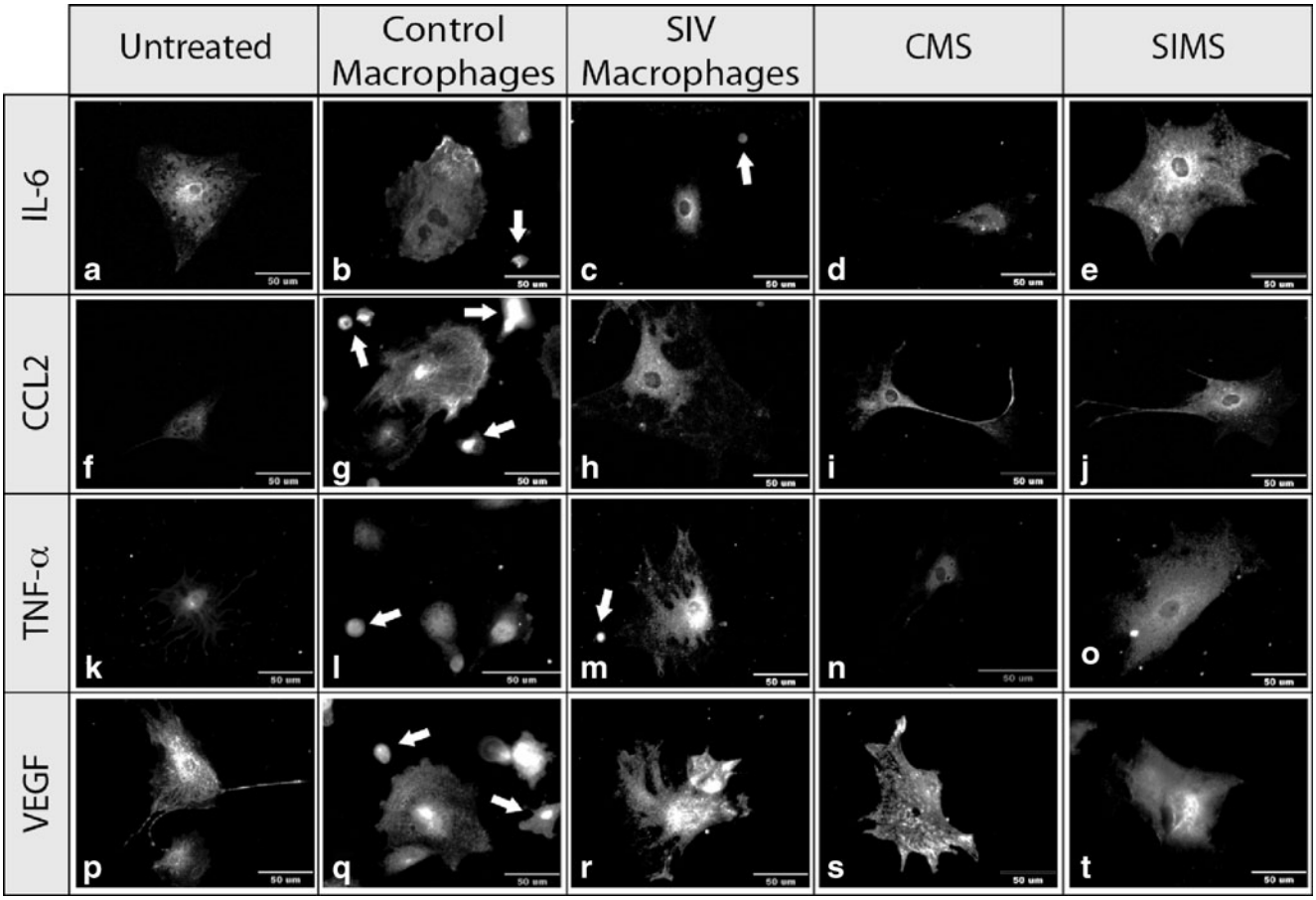

Fig. 4 Visualized expression of cytokines in cultured microglia. IL-6 production was detectable in all treatments (**a**–**e**). SIV-infected BMDM supernatant-treated (**e**) microglia produced abundantly higher levels, consistent with multiplex data. The presence of macrophages (*arrows*), either control (**g**) or SIV-infected (**h**), induced higher levels of CCL2 than did their respective supernatant (**i** and **j**, respectively), although there was increased expression over the untreated microglia (**f**). There was minimal expression of TNF-α by resting microglia in culture (**k**), which was increased in all four treatment groups. The most intense staining was in the macrophage treatment groups (**l**, **m**) with the lowest being control BMDM supernatant (**n**). VEGF was expressed in each of the conditions, including untreated microglia (**p**). The presence of macrophages (*arrows*), either control (**q**) or SIV-infected (**r**), induced minimally increased expression of VEGF. Cell-free supernatant from control (**s**) or SIV-infected (**t**) macrophages induced a punctate expression pattern indicative of increased secretion

were infected with SIV (m) or not (l), consistent with multiplex data (Fig. 3j). CMS (n) and SIMS (o) also increased the levels of TNF-α expressed over baseline, although there appear to be differences in the patterns of expression from numerous, disseminated, fine vesicles for SIMS treatment through very intense, but focal, staining with CMS.

VEGF was expressed by all microglia stained (Fig. 4p–t). Intense foci of staining were apparent with either CMS or SIMS treatment, with a more diffuse pattern evident when BMDM were incubated with microglia.

These data confirm the multiplex results (Fig. 3), with similar levels of cytokines expressed by brefeldin A-treated microglia following co-incubation with control or SIV-infected BMDM. The data also confirm that incubation with SIMS induced very high levels of IL-6. The BMDM added to the cultures were noted to express IL-6 (Fig. 4b, c), CCL2 (g), TNF-α (l), and VEGF (q). Therefore, it is probable that, in addition to microglia secreting cytokines, BMDM were contributing to the cytokine levels in Fig. 3.

Conclusion

Microglia cultured from normal rhesus macaques have a morphology similar to those observed in vivo (Figs. 1 and 2) and secrete low levels of pro-inflammatory cytokines (Figs. 3 and 4). On incubation with either control or SIV-infected BMDM, microglia take on an amoeboid morphology (Fig. 2b, c) with alterations in AIF-1 expression reflecting alterations in actin cytoskeleton. This coincides with increased expression of pro-inflammatory cytokines (Figs. 3 and 4). There was no significant difference between levels of cytokines secreted by control BMDM and SIV BMDM.

Our data suggest that microglial activation in the brain may be due to contact with macrophages. This response is not changed by SIV infection of macrophages. However, SIV-infected macrophages appear to secrete factors that augment the microglial response. This suggests that the neuroAIDS response relies on a two-pronged initiation: a macrophage–microglia contact that is not related to the infection status of the macrophage. This is supplemented by microglia responding to soluble mediators released from SIV-infected macrophages.

Combined, these results suggest that further examination of cultured microglia cells will provide a useful tool to examine morphological changes, cytokine expression, and cell-to-cell interactions in glial cells. It will be of interest to determine if these changes are conserved in other neuroinflammatory diseases such as Lyme neuroborreliosis, multiple sclerosis, West Nile virus, or eastern equine encephalitis.

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