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Microarray analysis of IFN-γ response genes in astrocytes

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Abstract

IFN-gamma (IFN-γ) has been shown to activate astrocytes to acquire immune functions. In this study the effect of IFN-γ on murine astrocytes was investigated via microarray analysis. The activating effect of IFN-γ on the astrocyte transcriptome showed predominance toward pathways involved in adaptive immunity, initiation of the immune response and innate immunity. Previously unknown astrocytic genes expressed included members of the p47 GTPases and guanine nucleotide binding protein (GBP) families. Down-regulatory effects of IFN-γ stimulation were confined to pathways involved in growth regulation, cell differentiation and cell adhesion. This data supports the notion that astrocytes are an important immunocompetant cell in the brain and indicate that astrocytes may have a significant role in various infectious diseases such as Toxoplasmic Encephalitis and neurological diseases with an immunological component such as Alzheimer's and autoimmune disorders.

Keywords

Astrocytes; Gamma interferon; Cytokines; Toxoplasmic Encephalitis; p47 GTPases

1. Introduction

Astrocytes are the predominant glial cell in the central nervous system (CNS) and have diverse physiological functions in the CNS. For instance, astrocytes secrete neurotrophic factors, regulate extracellular pH and K^+ levels, and metabolize neurotransmitters and thus are an important to maintaining neuronal functions (Benveniste, 1998). Astrocytes also help maintain the blood brain barrier (BBB) and the glial limitans and thus are important in maintaining homeostasis in the undamaged CNS (Wolburg and Risau, 1995). Increasingly astrocytes have also become to be appreciated as functioning as an immunocompetant cell in the CNS (Dong and Benveniste, 2001). Gamma interferon (IFN-γ) for example induces increases in cell adhesion molecules and MHC II expression in astrocytes indicating that astrocytes may help regulate recruitment of T cells into the brain and be capable of acting as an antigen presenting cell in the brain. IFN-γ also induces astrocytes to secrete proinflammatory cytokines such as IL-6 leading to the suggestion that IFN-γ activated astrocytes may contribute to immunopathology in the brain during infectious diseases and autoimmune disorders such as HIV associated dementia, Alzheimer's Disease and multiple sclerosis (Minagar et al., 2002; Mucke and Eddleston, 1993; Dong and Benveniste, 2001).

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2006.02.015.

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More recently however it has been suggested that cytokine activated astrocytes also promote the recovery and repair of CNS function by producing trophic factors for neurons and oligodendrocytes, acting as free radical and excess glutamate scavengers, actively restoring the blood – brain barrier, promoting neovascularization, restoring CNS ionic homeostasis, promoting remyelination and stimulating neurogenesis from neural stem cells (John et al., 2003; Liberto et al., 2004). These studies indicate that astrocytes have important and variable roles in the immune response in the CNS infection.

IFN-γ is a pleiotrophic cytokine that induces a large number of different cellular programs that collectively regulate the immune response. For example IFN-γ stimulates bacteriocidal activity of phagocytes, antigen presentation via Class I and Class II major histocompatibility complex (MHC) molecules, orchestrates leukocyte–endothelium interactions and has effects on cell proliferation and apoptosis (Boehm et al., 1997). Over 500 IFN-γ response genes have been identified although it is estimated that IFN-γ regulates expression of over 1200 genes (Boehm et al., 1997; MacMicking, 2004). The functional significance of many of these genes however, remains unknown. IFN-γ has potent activating effects on professional immune cells such as macrophages that play a key role in defense against infectious agents. In the brain, microglia are the main immune effector cell and IFN-γ activates a variety of immune effector functions such as nitric oxide production, reactive oxygen species and antigen presentation via MHC Class II pathway (Aloisi et al., 2000; Aloisi, 2001). Recent studies have found that astrocytes also acquire some of these immune effector functions (Ashner, 1998; Carson and Sutclifte, 1999; Benveniste, 1998). Additionally our previous studies with the intracellular protozoan, *Toxoplasma gondii*, found that astrocytes stimulated with IFN-γ were able to inhibit growth of this intracellular pathogen by a mechanism that was found to be independent of all known anti-microbial mechanisms including nitric oxide, reactive oxygen species and tryptophan degradation (Halonen et al., 1998; Halonen and Weiss, 2000). This anti-microbial mechanism remains unknown although it has been shown to be dependent upon IGTP, a newly described IFN-γ response protein (Halonen et al., 2001). Thus ours and numerous other studies, indicate astrocytes are an important immune effector cell in the CNS. The IFN-γ response genes and the phenotypic changes induced by IFN-γ in astrocytes however, remains less well understood.

Oligonucleotide arrays have been used to study the differential expression of IFN-γ induced gene expression in a variety of cell types. In this study we have used a microarray approach to characterize the IFN-γ response genes in astrocytes and to further study the unknown IFN-γ induced inhibition of *T. gondii* in astrocytes. In this study, astrocytes were stimulated with 100 U/ml IFN-γ for 72 h, as previous studies found the IFN-γ induced inhibition of *T. gondii* required a stimulation of 48–72 h. To obtain a comprehensive list of the interferon-γ response genes in astrocytes, the Affymetrix Mouse 430 2.0 GeneChip with 45,101 probe sets which covers the entire murine genome, was used to assay for differential gene expression in primary murine astrocytes which were stimulated with IFN-γ. Over 4000 genes were differentially expressed in astrocytes in response to IFN-γ. Of these IFN-γ response genes 754 known genes were differentially expressed by a factor of 1.5. Many of the IFN-γ response genes identified in astrocytes in this study have been previously identified as IFN-γ response genes but many previously unidentified IFN-γ response genes and genes of unknown function were also found in this study. Significantly, the identification of IFN-γ response genes in astrocytes provides informative insights into the mechanism(s) of IFN-γ action and the role of astrocytes as a potent immune effector cell in the CNS.

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2. Methods

2.1. Primary murine astrocyte culture

Murine astrocytes from C57BL/6 × SV 129 mice were cultivated from the brains of neonatal (less than 24 h old) mice. Murine pups were sacrificed, the brains removed from the cranium, the forebrain dissected and the meninges removed. The tissue was minced and incubated in 0.25% trypsin for 5 min at 37 °C. After 5 min, the trypsin was inactivated with a solution containing Dnase and soybean trypsinase inhibitors, and the tissue was further disrupted by trituration in a 20-ml pipette. The dissociated cells were filtered through a 74 μm Nitex mesh, centrifuged at 200 ×*g*, suspended in growth medium at a concentration of 10^6 cell/ml, and plated onto poly-L-lysine coated dishes. Astrocytes were maintained in endotoxin-free minimal essential medium (BRL-GIBCO) supplemented with MEM Vitamins, MEM amino acids, glutamine (20 mM), and 20% fetal bovine serum. The growth medium (S-20) was changed every 3 days. After 7 days in vitro, a confluent layer of 1×10^4 cells/cm^2 of astrocytes is reached. By this method, cells were found to be >95% astrocytes, as judged by positive staining for glial fibrillary acidic protein. Cultures contained <5% microglia, as identified by staining with the lectin BS1-B4 (Sigma L-2895). Astrocytes were dissociated in trypsin–EDTA, replated onto poly-L-lysine coated coverslips or 24 well plates at 10^4 cells/cm, cultured for 7 to 10 days after replating and then stimulated with IFN-γ as described below.

2.2. IFN-γ treatment and experimental design

Astrocytes from three separate murine astrocytes preparations were used for these experiments. From each astrocyte preparation, astrocytes were cultured in S-20 media only or treated with 100 U/ml IFN-γ for 72 h, washed in PBS and then harvested for RNA as described below. Recombinant murine IFN-γ was purchased from Endogen (Pierce Biotechnology, Rockford, IL).

2.3. RNA extraction

Total RNA was extracted from murine astrocyte cultures that were cultured as described above in Trizol and RNA isolated as per the manufacturer's instructions. The amount of total RNA extracted was measured by spectrophotometry (GeneQuantPro) and RNA quality was assessed using a 1% agarose gel containing ethidium bromide. Only samples of high quality (absorbance at 260/280 greater than 1.8 and absence of signs of RNA degradation) were included in the study. The total RNA from each astrocyte sample was subdivided into two, with one half used for the preparation of target cRNA for microarray analysis and one half for validation of the relative gene expression levels by reverse transcriptase PCR (RT-PCR).

2.4. Preparation of 'target cRNA' for microarrays

Target cRNA for microarray analysis was prepared following the manufacturer's protocol (http://www.affymetrix.com). In brief, total RNA (approximately 5 μg per sample) was used to synthesize double-stranded cDNA by reverse transcription using a T7-oligo (dT) primer in the first strand cDNA synthesis reaction, followed by RNase-mediated second-strand cDNA synthesis. Subsequently, biotin-labeled cRNA was prepared from double-stranded cDNA by in vitro transcription using T7 RNA polymerase. The biotinylated cRNA was subsequently treated with RNeasy clean-up kit (Qiagen) to remove any unincorporated biotinylated ribonucleotides from the cRNA samples and quantitated. Purified cRNA (15 μg) was then fragmented to produce cRNA of 35 to 200 base fragments. The fragmented target cRNA (10 μg) was mixed with 200 μl of hybridization cocktail (100 mM 2-morpholino-ethanesulfonic acid (MES), 1 M NaCl, 20 mM ethylenediaminetetraacetic acid, 0.01% Tween 20, 0.1 mg/ml herring sperm DNA, 500 g/ml acetylated bovine serum

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albumin) and was hybridized to the Affymetrix murine genome (MOE 430 2.0) at 45 °C for 16 h in a rotisserie oven at 60 rpm. Following hybridization, the target cRNA was removed; the arrays were first washed in 6× SSPE at 25 °C and then washed again with 100 mM MES, 0.1 M NaCl, and 0.01% Tween 20 at 50 °C. In the following step, the arrays were stained with streptavidin IgG (3 μg/ml), followed by a second staining with streptavidin phycoerythrin. Finally the arrays were rinsed in 100 mM MES, 0.1 M NaCl and 0.01% Tween 20 at 50 °C before the scanning step in the Gene Array scanner (Affymetrix, Santa Clara, California, USA).

2.5. Microarray data analysis

Data were analyzed by using GeneSpring Suite 7 software. Data was normalized using CEL files followed by RMA normalization, median polishing and the raw data filtered. Group comparison between baseline and stimulation was performed using the data of 3 arrays of unstimulated and 3 arrays of IFN-γ-stimulated astrocytes. The normalized array intensities were employed to compare expression intensities between unstimulated and IFN-γ stimulated astrocyte samples. Only genes that received present calls, respectively, in all chips assayed were included. The three replicates were averaged and genes that were statistically significant ($p < 0.05$; Student's *t*-test) were included in this analysis. Only genes that showed fold change values above or below 1.5 were used in this analysis.

2.6. Validation of the relative gene expression values using RT-PCR

Microarray data were validated using reverse transcriptase PCR (RT-PCR) for the 6 p47GTPases, IGTP, IRG, LRG, TGTP, IIGP and GTPI. Gene expression of these 6 genes was determined for astrocytes treated with media alone or stimulated with 100 U/ml IFN-γ for 72 h. Total RNA was isolated from each treatment group and then used in RT-PCR assay using SYBR green following the manufacturers protocol. The primer sequences for the 6 p47 GTPases were as follows: IGTP forward primer was CTCATCAGCCCGTGGTCTAA and IGTP reverse primer was CACCGCCTTACCAATATCTTCAA; IRG-47 forward primer was AGCAGATGAATCCGCTGATGT and IRG-47 reverse primer was CGTGGAAATTGGGTGTCCC; IIGP forward primer was CAGGACATCCGCCTTAACTGT and IIGP reverse primer was AGGAAGTAAGTACCCATTAGCCA; GTPI forward primer was ATCCGGGATAGTCTTCAGAAGG and GTPI reverse primer was AGGGAGGTCTTTTTGTAGTGTCT; LRG-47 forward primer was TTTCATCAATGCACTTCGAGTCA and LRG-47 reverse primer was AATCCAGGTAAGTCCCACAGC; TGTP forward primer was TGGGACCACTAACTTCACACC and TGTP reverse primer was GGCCAGTTGTGCATCATTTTC. Actin was included as an internal control for a housekeeping gene whose expression should not change. The forward primer used for actin was GTGAGATTGTGCGCGACATC and the reverse primer was GGCAACGGAAACGCTCATT. Gene expression values were determined by calculation of the relative expression ratio from real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control as described by Pfaffl (2001).

3. Results

3.1. IFN-γ response genes in murine astrocytes

Of the possible 45101 genes represented on the Affymetrix Murine 430 2.0 gene chip, 4006 genes were differentially expressed in IFN-γ stimulated astrocytes. Of these 4006 IFN-γ response genes, 1192 genes were expressed by a factor of 1.5 or greater. Approximately one-fourth of these genes were of unknown identity and removed from the data set. The remaining 754 genes were identified as differentially expressed by a factor of 1.5 fold, with

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443 genes up-regulated and 314 genes down-regulated (Table 1A). Amongst both the up and down-regulated genes a large number of genes were found to change by 1.5 to 2.0 fold and although this change was modest, many of these genes were amongst the highest significance ($p < .001$) and were thus included in further analysis. The up and down regulated IFN-γ response genes were functionally classified using GO Biological Pathways as listed in Table 1B and C. A complete listing of all the IFN-γ response genes (754) expressing a 1.5 fold change, identified in this study, is included in Appendix 1 (supplementary material).

The up-regulated IFN-γ response genes were involved in several biological pathways but the predominant pathway was the immune response comprising about 27% of the upregulated genes. Other pathways represented were apoptosis, intracellular and extracellular transport, lipid/steroid metabolism and endocytic/exocytic pathways with each of these representing less than 10% of the upregulated genes (Table 1B). A large percentage of these up-regulated genes were either of unknown function (11%) or genes with cellular functions that were not clearly defined into distinct biological pathways (33%). Approximately one tenth of the up-regulated genes (65) were induced in excess of 10 fold and were considered de novo expressed genes. This selected list of up-regulated genes is listed in Table 2. These strongly up-regulated genes are almost exclusively involved in immune functions with MHC Class I and II molecules and accessory molecules such as the invariant chain and subunits of the proteosome, p47 GTPases, guanine binding proteins, chemokines, HIN-200 proteins and the transcription factors, STAT 1 and IRF-1, all strongly induced. Also amongst the other strongly up-regulated genes were a few non-immunologically related genes such as calbindin, a calcium binding protein involved in locomotion, and two genes thought to be involved in apoptosis. The highest of these de novo upregulated genes, were a MHC Class II gene, and Iigp and TGTP, members of the p47GTPase family, all of which were induced by approximately 200-fold (Table 2).

The down regulated IFN-γ response genes were involved in a variety of pathways including regulation of cell growth and differentiation, metabolic pathways, immune response, ion transport, cell adhesion, cell signaling, endocytic/exocytic pathways and lipid/steroid pathways (Table 1C). Immune response genes were present amongst the down-regulated genes but immune response genes comprised only 10% of the down-regulated genes as compared to the 27% of the upregulated genes. The most significant IFN-γ induced down-regulated genes, expressing at least a 2-fold change, are listed in Table 3. Amongst the down regulated genes, the fold change was much less as compared to the up-regulated genes with the greatest fold change expressed by the macrophage scavenger protein, down regulated approximately 6×, and with most of the other down regulated genes in the range of a 2–3 fold change. The genes expressing the greatest degree of down regulation were the macrophage scavenger receptor 2, exhibiting an approximately 6-fold change, the related gene, macrophage scavenger receptor 1, exhibiting a 3.1 fold change and the mannose receptor, exhibiting a 5 fold change. A few immune response genes are included in this list such as, a complement component and the mannose receptor, but in general the most strongly down-regulated genes were involved in cell adhesion and cell proliferation and differentiation pathways (Table 3).

3.2. Genes up regulated by IFN-γ stimulation involved in host defense

Amongst the up-regulated genes, the majority of genes were involved in the immune response and these were also the most strongly induced IFN-γ response genes (Table 2). A functional classification of these up-regulated host defense genes is listed in Table 4. The largest proportion of the host defense genes were involved in MHC Class I and Class II pathways. Genes involved in MHC Class I pathway, for example, include MHC Class I molecules, β_2 microglobulin, and accessory molecules such as the peptide transporter

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molecule, TAP1, ubiquitin molecules and several components of the proteosome. Likewise genes involved in the MHC II pathway include MHC II molecules, MHC II accessory molecules such as Ia-associated invariant chain and DMa, and the costimulatory molecules, CD80 and CD86. As expected the IFN-γ induced transcription factors, STAT 1, interferon response factor (IRF), and interferon consensus stimulated binding protein (ICSBP) were also upregulated. Some chemokines and cytokines or cytokine receptors, were also found to be upregulated. Also notable were the GTP binding proteins, including the p47 GTPases and guanine binding proteins, which are recently described IFN-γ response genes but whose function(s) are not well understood. Additionally some genes involved in anti-viral defense (4%) such as 2′,5′-oligoadenylate synthetase and to a lesser extent other anti-microbial defense genes such as those involved in tryptophan metabolism and nitric oxide and reactive oxygen species (1%) were also represented in the IFN-γ induced astrocyte transcriptome. Finally a large number of genes (27) were categorized as having other immune functions. This list included genes such as CD1 antigen which is involved lipid antigen presentation, the adhesion molecules, ICAM-1 and VCAM-1, the toll-like receptor 3, which is involved in innate immunity and some genes whose immune function(s) are not characterized. A complete listing of the IFN-γ Response Up-regulated Genes Involved in the immune response is included in Appendix 2 (supplemental material).

3.3. p47 GTPases and GBP protein families

The p47 GTPases and guanine binding protein (GBP) families are two newly described families of IFN-γ response proteins. Members of both the p47 GTPase and GBP proteins were amongst the highest IFN-γ up-regulated genes in astrocytes. For example all six known members of the p47 GTPase and three of the guanine binding proteins, GBP1, GBP2, GBP4, are listed amongst the most strongly IFN-γ response genes in Table 2. Of the six p47 GTPases, the most strongly induced were IIGP, TGTP and IRG-47, exhibiting a 206, 195 and 100 fold change respectively and IGTP, GTPI and LRG-47 exhibiting a 44, 30 and 15 fold change respectively (Table 2). The up-regulation of all 6 of these p47 GTPases in IFN-γ treated astrocytes was validated by RT-PCR (Table 5). RT-PCR confirmed that all of the p47 GTPases were significantly upregulated upon IFN-γ stimulation with IIGP and TGTP the most strongly upregulated and LRG-47 having the lowest change in expression, as was found from the microarray analysis. The fold change expression values detected by RT-PCR were also comparable to those detected in the microarray analysis.

3.4. Other important pathways

In addition to the immune response genes, the initial analysis of the biological pathways represented amongst the IFN-γ response genes indicated that genes involved in endocytic/exocytic trafficking and lipid/steroid pathways were differentially regulated by IFN-γ in astrocytes (Table 1B and C). The endocytic/exocytic pathway was of interest given the strong upregulation of the p47 GTPases, which are thought to be involved in regulation of vesicular trafficking and the lipid/steroid pathway was of interest due to the recent studies suggesting *T. gondii* requires sterols from its host cells (Coppens et al., 2000). Thus the effects of IFN-γ on the vesicular and lipid/steroid pathways were investigated further.

A small number of genes involved in vesicular pathways were found to be differentially regulated by IFN-γ in astrocytes (Table 6). Amongst these genes were two rab genes, Rab 19 and Rab 6B, which were upregulated and down regulated respectively. Also amongst the IFN-γ upregulated genes were a sorting nexin, synapsin II and a vacuolar sorting protein 54 (Vps54) and amongst the down-regulated genes, stabilin which is involved in sorting in the vesicular pathway and the adaptor protein, AP-1, amongst others. All of these genes were only modestly affected (fold change values from 3.4 to 1.5).

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Of the lipid related genes, phospholipase A1, increased by more than 9-fold and was the gene exhibiting the greatest induction (Table 7). The genes involved in cholesterol metabolism which were moderately upregulated included StAR-related lipid transfer (START) domain containing 5, sterol O-acyltransferase I, arrestin domain containing 4 while other genes involved in cholesterol metabolism such as macrophage scavenger receptors 1 and 2, Neiman–Pick type C1, and a member of the ABCA transporter family, were found to be down regulated.

4. Discussion

This study found that IFN-γ stimulation results in highly selective expression of astrocyte genes. More than 45,101 gene sequences were examined and of these, 4006 genes were differentially expressed in IFN-γ stimulated astrocytes. A total of 754 genes were identified that were induced by IFN-γ by a factor of 1.5 with 443 up-regulated and 314 down-regulated. The majority of the IFN-γ response genes up-regulated were involved in the immune response and included chemokines, cytokines and components of the MHC I and II mediated antigen presentation pathways. The most dominant up-regulated genes were the molecules involved in MHC II and MHC I presentation pathways. Additionally all six members of p47 GTPases and several members of the GBP family were also amongst the most strongly induced IFN-γ response genes in astrocytes. In this study genes at only one time point, a 72 h stimulation, were examined and thus only late IFN-γ induced responses could be detected. However, while microglia are well known to play a crucial role in the immune responses in the CNS, these results contribute to the considerable evidence that Th1 activated astrocytes, the most numerous glial cell in the CNS, are also a significant component of the immune response in the CNS.

The astrocytes used in this study were primary astrocytes derived from mouse brain. Using this procedure we typically obtain cultures that are greater than 95% astrocytes and less than 5% microglia cells. Thus while some microglia are contained within our sample and it is possible some of the genes induced by IFN-γ in our microarray experiments may be derived from microglial cells and not astrocytes, microglia comprise only a relatively small percentage of the total culture. Perusal of the dataset for macrophage and microglial markers found the macrophage marker CD68 was present, but at low levels while the macrophage markers, MRP8 and MRP14, and the microglia marker, CD11b, were not present. One can therefore conclude that although some microglia cells may be present they are at low levels and the genes reported in this dataset are predominantly derived from astrocytes. In addition, a recent microarray of the IFN-γ response genes in microglial cells found a distantly different array of genes than our study further indicating that the IFN-γ response genes detected in our study are primarily derived from astrocytes (Moran et al., 2004).

IFN-γ binds to its receptor and initiates a signaling cascade, involving the Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs) (Platanias, 2005; Hoey and Grusby, 1999). The STAT 1 transcription factor and secondary interferon regulator factors, IRF-1, IRF-2, and ICSBP, leads to the induction of IFN-γ response genes that encode for proteins that mediate the IFN-γ response. The most important IFN-γ stimulated cellular programs include MHC I and MHC II pathways, which are involved in antigen presentation, nitric oxide and respiratory burst, which have direct anti-microbial activities and molecules involved in the translational inhibition of viral replication. Other known IFN-γ response genes included other components of immunity such as chemokines, complement components, FcRs and toll like receptors (TLRs). Many of these known IFN-γ response genes involved in the immune response were detected in astrocytes in this study. For example, OAS and PKR, both involved in anti-viral activities, MHC Class II molecules and molecules involved in the endogenous antigen presentation pathway such the invariant

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chain, Ii and DMa, and MHC Class I genes and molecules in the Class I antigen presentation pathway such ubiquitin pathway and proteasome components and antigen transporters, were found to be significantly up-regulated by IFN-γ. Some cytokines such as IL-7 and IL-15, were produced by IFN-γ stimulated astrocytes in our study although notably IL-6 which is the cytokine commonly found to be one of the most strongly upregulated in astrocytes was not induced in our study. The chemokines, RANTES/CCL5, monocyte chemoattractant protein 1(MCP-1)/CCL2 and interferon-γ-inducible protein (IP-10)/CXCL10, all of which are proinflammatory serving to attract monocytes and T cells to the site of inflammation and known to strongly induced in activated astrocytes, were also found to be upregulated (Dong and Benveniste, 2001). Additionally, the adhesion molecules, ICAM-1 and VCAM-1, molecules important in T cell infiltration into the CNS were also upregulated. The production of chemokines and adhesion molecules support the idea that activated astrocytes play a crucial role in T cell infiltration into the CNS and may be important in diseases such as multiple sclerosis, Alzheimer's Disease and infectious diseases such as Toxoplasmic Encephalitis. Many of the immune response genes identified as upregulated by IFN-γ in this study have been reported in other studies (Dong and Benveniste, 2001; Mucke and Eddleston, 1993; Carpentier et al., 2005). Results from this study confirm the numerous studies that indicate that IFN-γ activated astrocytes acquire a diverse array of immune effector functions, including acquiring capabilities of presenting antigens via both Class I and Class II pathways, enhancing the intracerebral immune response by regulating lymphocyte trafficking and anti-viral and antimicrobial activities.

One of the most strongly IFN-γ upregulated pathways in astrocytes in this study was the MHC II pathway. The IFN-γ induced increase of MHC II expression is not surprising, as this has been reported in numerous other studies. However, the role of astrocytes as functional APCs in the adaptive immune system has been controversial. This is partly due to discrepancies from both in vitro and in vivo studies about the ability of astrocytes to express the accessory molecules, CD80 and CD86 (B7.1 and B7.2 respectively in mice). Some in vitro studies in mice for example, have found that IFN-γ stimulated astrocytes expressed both B7.1 and B7.2 while other studies found IFN-γ stimulated astrocytes expressed neither B7.1 or B7.2 (Aloisi et al., 1998; Girvin et al., 2002; Nikcevich et al., 1997; Soos et al., 1998). Likewise some in vivo studies have found IFN-γ induces B7.2 expression on astrocytes while others found expression of neither B7.1 nor B7.2 on astrocytes (Issazadeh et al., 1998; Cross and Ku, 2000). Reports on the capability of astrocytes to function as APCs are also contradictory with some studies reporting astrocytes are capable of presenting antigen to naïve, Th1, or Th2 $CD4^+$ T cells while others have reported astrocytes are inefficient at processing or presenting antigen (Fierz et al., 1985; Aloisi et al., 1995, 1998; Nikcevich et al., 1997; Tan et al., 1998; Carpentier et al., 2005). In this study we found IFN-γ stimulated astrocytes, in addition to MHC Class II molecules, exhibited strong up-regulation of both CD80 and CD86 indicating astrocytes are capable of stimulating naïve T cells. In addition to MHC II, CD80 and CD86 expression, we also found the other components of MHC II antigen presentation pathway such as Ii and HLA-DM were also strongly upregulated, indicating that IFN-γ stimulated astrocytes are fully equipped to function as an APC. Our results are similar to a recent in vitro study of IFN-γ and TNFα stimulated astrocytes that examined expression of MHC II, CD80, CD86, and CD40, via FACS analysis and found upregulation of all of these molecules (Carpentier et al., 2005). The issue of expression of MHC II in conjunction with CD80 and CD86 is important because the presence of these co-stimulatory molecules indicate astrocytes could stimulate T cells while their absence would indicate astrocytes might promote TH2 responses and/or apoptosis of T cells (Dong and Benveniste, 2001). This issue ultimately remains to be resolved via in vivo studies. However, ours and other studies indicate astrocytes are potentially, fully capable of activating TH1 cells which may be important in many diseases,

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as astrocytes, although less efficient at activating T cell than microglia, are the predominant glia cell in the brain and thus potentially may contribute significantly to T cell activation.

In addition to immune response genes involved in antigen presentation pathways, some of the other most strongly induced genes which have not been identified previously were members of the two recently described families of GTPases proteins, the p47 GTPases and the guanine binding proteins (GBPs). The GBP family ranges in size from 65 to 67 kDa and includes two members in humans, GBP1 and GBP2, and 5 members in mice, GBP1–GBP5 (Boehm et al., 1998; Nguyen et al., 2002). The p47GTPase family is comprised of six members called, IRG-47, LRG-47, TGTP, GTPI, IGTP and IIGTP. Both families are strongly induced by IFN-γ but the GBP family requires IRF-1 whereas the p47 GTPases do not, indicating that the GBP family are secondary response genes whereas the p47GTPases are primary response genes. The function(s) of the GBPs is not yet clear but they are thought to be involved in resistance to viruses (Anderson et al., 1999). The function of the p47 GTPases is also not clear but several have been found to be membrane bound and localize to the ER, and several studies have indicated that they mediate host resistance by regulating pathogen survival in host cells (Halonen et al., 2001; Taylor et al., 2004; MacMicking, 2004). Some of the p47 GTPases (IGTP, LRG) regulate phagosome related functions and others (TGTP) mediate anti-viral resistance by regulating apoptotic pathways of infected host cells (Taylor et al., 2004). Our previous studies found IGTP to be important in the resistance to IFN-γ induced inhibition of *T. gondii* in astrocytes by an unknown mechanism (Halonen et al., 2001). In this study we found all six of the p47 GTPases and four of the five GBPs were strongly induced in astrocytes. Although the function of these IFN-γ response genes is not clear, given what is known about these proteins, data from this study imply that IFN-γ stimulated astrocytes have robust undescribed anti-microbial mechanisms to inhibit growth of a diversity of pathogens including compartmentalized pathogens as well as cytosolic pathogens such as viruses.

Given the effect of IFN-γ on p47GTPases and their suspected role in vesicular trafficking, we investigated whether genes involved in vesicular pathway were affected by IFN-γ. Although only a few genes were differentially regulated by IFN-γ in astrocytes, those that were identified were involved in regulating the flow of membrane trafficking through the vesicular pathway. Two rab genes, rab19 and rab 6B, were found to be up and down regulated, respectively. Additionally, sorting nexin 10, a member of the nexin family, which regulates membrane trafficking between endosomal organelles, was upregulated (Carlton et al., 2004; Worby and Dixon, 2002; Zhong et al., 2002). The other genes of interest involved in the vesicular pathways that were upregulated were VPS54, required for retrograde transport of Golgi membrane proteins from endosomal compartments and synapsin II which in involved in post-trans-Golgi trafficking pathways (Bustos et al., 2001, Maienschein et al., 1999; Conibear and Stevens, 2000). Additionally the genes, stabilin-1, Rab6B, and the adaptor complex, AP-1, which are all involved in vesicular trafficking from the Golgi to the endosomes, were all down regulated (Kzhyshkowska et al., 2004; Opdam et al., 2000; Robinson, 2004). Thus although the effect on vesicular trafficking found in this study was modest, collectively these genes do suggest that in astrocytes IFN-γ may affect genes that are involved in regulation of membrane trafficking events in the vesicular trafficking pathway. In support of this idea, IFN-γ has been shown to slow rate of membrane trafficking in macrophages (Tsang et al., 2000). Interestingly one consequence of slowed membrane trafficking in activated macrophages was a prolonged exposure of incoming molecules to an acidic nonlysosomal compartment, a condition which was suggested to facilitate antimicrobicidal mechanisms and/or antigen processing. It would be interesting to investigate the effect of IFN-γ on membrane trafficking rates in astrocytes and specifically to investigate if these effects on membrane trafficking might be related to the IFN-γ induced inhibition of *T. gondii* in astrocytes. IFN-γ induced changes in vesicular trafficking may also

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have affects on the MHC I and II antigen processing and presentation pathways and possibly be involved with the p47 GTPases.

The other pathway of interest affected by IFN-γ was related to sterol/cholesterol metabolism. Several genes such as sterol O-acyltransferase 1, and StAR-related lipid transfer (START) domain containing 5, and arrestin domain containing 4, which are involved in intracellular cholesterol trafficking and metabolism, were up-regulated while Nieman Pick protein, involved in cholesterol efflux from endosomes and lysosomes, and the low density receptor-related protein 2 and SR-A and SR-B, receptors for low-density lipoprotein, were found to be down-regulated in response to IFN-γ in astrocytes. IFN-γ is well known to inhibit reverse cholesterol transport in macrophages by modulating type A and B scavenger receptors, decreasing ABCA1, a cholesterol efflux transporter and increasing cholesterol ester accumulation via up-regulating acyl coenzyme A : cholesterol-*O*-acyltransferase (ACAT) activity (Wang et al., 2002). In macrophages modulation of these genes related to cholesterol metabolism results in an accumulation of cholesterol within macrophages resulting in foam cells and promotes atherosclerosis (Christie et al., 1996). While the changes in cholesterol genes identified in astrocytes in this study were modest, these results suggest IFN-γ may also affect cholesterol metabolism in astrocytes. We did not find changes in ACAT, apolipoproteins or other cholesterol related genes, although changes in these genes may be early events that would not be detected in this study. Astrocytes are known to be important in cholesterol metabolism in the brain but IFN-γ induced changes in cholesterol metabolism in astrocytes have not been well studied. Effects of IFN-γ on cholesterol metabolism in astrocytes may play a role in inflammatory diseases in the brain such as Alzheimer's Disease and deserves further study.

Astrocytes represent the most abundant yet least understood cell in the CNS. In this study we have examined the IFN-γ response genes in astrocytes via microarray analysis. Our results have confirmed previous studies indicating IFN-γ stimulated astrocytes acquire a variety of immune functions. Additionally we have identified previously unidentified IFN-γ response genes such as the p47GTPases in astrocytes that indicate astrocytes may have additional immune functions against infections that are not yet understood. Additionally some interesting changes in cholesterol and vesicular trafficking pathways were detected in IFN-γ stimulated astrocytes that may have implications in resistance to infectious agents such *T. gondii* and in the pathogenesis of neurological diseases such as Alzheimer's Disease. In this study genes at only one time point, a 72 h stimulation, were examined and thus only late IFN-γ induced responses could be detected. Future studies looking at earlier time points and analysis of the early IFN-γ response genes in astrocytes may yield useful information about role of the p47GTPases and these other pathways. Results from this study contribute and add to knowledge of astrocytes capabilities as immune effector cells and to the general understanding of the diverse phenotype of this important glial cell of the CNS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

IFN-γ response genes in murine astrocytes

1A. Overview of IFN-γ response genes in murine astrocytes

Number of genes differentially expressed	4006
Number of known genes differentially expressed 1.5 fold	754
Number of genes up-regulated	443
Number of genes down-regulated	314

Samples were run in triplicates. Numbers represent the genes significantly present in all replicates ($p < 0.05$).

1B. Functional classification of the up-regulated IFN-γ response genes

Functional category	Percentage
Immune response	27%
Transcription factors and regulation	8%
Extracellular and intracellular transport	5%
Apoptosis	4%
Cell growth and maintenance	3%
Lipid/steroid metabolism	3%
Endocytic/exocytic pathway	2%
Intracellular signaling	2%
Glucose metabolism	1%
Cell adhesion	1%
Unknown function	11%
Miscellaneous biological functions	33%

1C. Functional classification of the down-regulated IFN-γ response genes

Functional category	Percentage
Cell growth and differentiation	15%
Metabolism	13%
Immune response	10%
Ion transport	8%
Cell adhesion	8%
Cell signaling	7%
Transcription factors and regulation	5%
Endocytic/exocytic pathway	4%
Lipid/steroid metabolism	2%
Other biological pathways	28%

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Table 2

Selected up-regulated IFN-γ response genes in murine astrocytes

Function	Description	GenBank no.	Fold change
MHC Class II	Histocompatibility 2, class II antigen A, alpha	BE688749	221.4
	Histocompatibiliy 2, class II H2-IA-beta chain	M15848	139.2
	Histocompatibility 2, class II antigen E beta	NM_010382	116
	Histocompatibility 2, class II antigen A, beta 1	NM_010379	103.4
	Histocompatibility 2, class II antigen E alpha	AF119253	85.3
	Histocompatibility 2, class II antigen A, alpha	AV018723	77.8
	Histocompatibility 2, class II, locus Mb1	NM_010388	32
	Histocompatibility 2, class II, locus DMa	NM_010386	24.1
	Histocompatibility 2, class II, locus Mb1	NM_010387	14.8
MHC II accessory molecule	Ia-associated invariant chain	BC003476	173.7
MHC Class I	Histocompatibility 2, Q region locus 7	M29881	104.3
	Histocompatibility 2, Q region locus 1	BC010602	67.5
	Histocompatibility 2, Q region locus 7	AK013097	37.3
	Histocompatibility 2, D region locus 1	M33151	29
	Histocompatibility 2, D region locus 1	M34962	15.5
	Histocompatibility 2, D region locus 1	M69068	12.6
	Histocompatibility 2, K region	L23495	10.8
	Histocompatibility 2, D region locus 1	L36068	10.6
	Histocompatibility 2, K region	BC011306	10.4
	Histocompatibility 2, D region locus 1	M86502	10.1
	Histocompatibility 2, T region locus 10	NM_010395	10.1
Protein degradation	Ubiquitin D	NM_023137	90.2
Proteasome	Proteosome subunit, beta type 9	NM_013585	40.4
	Proteosome subunit, beta type 8	NM_010724	21.1
	Proteosome subunit, beta type 9	AW048052	18.1
	Proteasome subunit, beta type 10	NM_013640	10.1
p47 GTPases	Iigp	BM239828	205.7
	T-cell specific GTPase (Tgtp)	NM_011579	195.3
	Irg-47	NM_008330	100.5
	Igtp	NM_018738	43.7
	Gtpi	NM_019440	29.3
	Lrg-47	NM_008326	15.2
Guanine binding proteins	Guanylate nucleotide binding protein 1	NM_010259	48.3
	Guanylate nucleotide binding protein 2	NM_010260	26.6
	Guanylate nucleotide binding protein 4	NM_018734	24.3
	Guanylate nucleotide binding protein 2	BE197524	23.3
Chemokines	Chemokine (C–X–C motif) ligand 10	NM_021274	70
	Chemokine (C–X–C motif) ligand 9	NM_008599	48.9
	Chemokine (C–C motif) ligand 5	NM_013653	20

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Function	Description	GenBank no.	Fold change
	Chemokine (C–X–C motif) ligand 11	NM_019494	12.7
Cytokines	Interleukin 18 binding protein	AF110803	23.2
Anti-viral	2′–5′oligoadenylate synthetase-like 2	BQ033138	29.3
Activation antigens	Macrophage activation 2, like	BM241485	75.3
	Lymphocyte antigen 6 complex, locus C	NM_010741	71.1
	Lymphocyte antigen 6 complex, locus A	BC002070	61.4
Transcription factors	Signal transducer and activator of transcription 1 (STAT1)	AW214029	30.5
	Interferon regulatory factor 1 (IRF1)	NM_008390	13.2
Transcription regulator	Interferon-induced protein with tetratricopeptide repeats 1	NM_008331	13.8
	Interferon-induced protein with tetratricopeptide repeats 3	NM_010501	10.5
HIN 200	Interferon activated gene 203	BC008167	12.6
	Interferon activated gene 203	NM_008328	12.6
	Interferon activated gene 205	M74124	13.1
	Interferon activated gene 205	AI481797	12.1
Cell signaling	SLAM family member 8	BC024587	10.2
	Suppressor of cytokine signaling 1	AB000710	10.6
Apoptosis	Serine (or cysteine) proteinase inhibitor, clade A, 3G	BC002065	63.2
Anti-apoptosis	Onzin	AF263458	12.6
Locomotion	Calbindin-28K	BB246032	11.8

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Table 3

Down regulated IFN-γ response genes exhibiting 2-fold change

Function	Description	GenBank #	Fold change
Cell adhesion	Glycosylation dependent cell adhesion molecule 1	M93428	5.6
	Neurotrimin	AF282980	2.6
	Integrin alpha 6	BM935811	2.5
	Cadherin 22	AB019618	2.3
	nel-like 2 homolog (chicken)	AI838010	2.2
	Protocadherin beta 20	NM_053145	2.1
	Monocyte to macrophage differentiation-associated 2	AV269411	2.1
	Immunoglobulin superfamily, member 4	BB333386	2.1
	Embigin	BG064842	2.1
	Claudin 2	NM_016675	2
Cell proliferation	Antigen identified by monoclonal antibody Ki 67	X82786	2.8
	Septin 3 (Sept3), mRNA	BQ176645	2.3
	Calmodulin binding protein 1	NM_009791	2.1
	Budding uninhibited by benzimidazoles 1 homolog	AF002823	2
Cell differentiation	Myeloid leukemia factor 1	AF100171	2.6
	Achaete-scute complex homolog-like 1 (Drosophila)	BB425719	2.3
	Monocyte to macrophage differentiation-associated 2	BC025064	2.2
	Growth associated protein 43	BB622036	2.1
Signaling pathways	Neurotensin receptor 2	NM_008747	3.1
	Neuron specific gene family member 2	NM_008741	2.7
	Epstein–Barr virus induced gene 2	BM242490	2.6
	G protein-coupled receptor 84	NM_030720	2.4
	Rac GTPase-activating protein 1	NM_012025	2
	Decorin	NM_007833	2
Immune response	Fc receptor, IgG, low affinity IIb	BM224327	4.7
	Complement component factor i	NM_007686	2.6
	Chemokine (C–X–C motif) ligand 4	NM_019932	4.3
	Chemokine (C–C motif) ligand 9	AF128196	2.8
	Mannose receptor, C type 1	NM_008625	5.1
Scavenger receptors	Macrophage scavenger receptor 2	BC016551	6.1
	Macrophage scavenger receptor 1	AA183642	3.1
Endocytic pathway	Stabilin 1	NM_138672	3.1
Transcription regulation	Myeloblastosis oncogene	NM_033597	4.5
	E2F transcription factor 7	BG069355	2.5
Prostaglandin metabolism	Hydroxyprostaglandin dehydrogenase 15 (NAD)	AV026552	3.2
Leukotriene metabolism	Leukotriene C4 synthase	NM_008521	2.2
Ion transport	Ion transport regulator 6	AB032010	3
Transporters	Solute carrier family 14 (urea transporter), member 1	AW556396	2.5
Enzymes	Glutaminase	BE983473	5.2

Function	Description	GenBank #	Fold change
	Inter-alpha trypsin inhibitor, heavy chain 3	NM_008407	2.5
	Protein phosphatase 1E (PP2C domain containing)	BB346082	2.2
	Phosphatidylethanolamine binding protein	AK008037	2
	Serine (or cysteine) proteinase inhibitor, clade A, member 3N	NM_009252	2
	Chitinase 3-like 1	BC005611	2
Metalloproteinase inhibitor	Tissue inhibitor of metalloproteinase 4	BI788452	2.3
Metalloproteinases	Matrix metalloproteinase 3	NM_010809	2.1
	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 15 (ADAMTS)	AV228731	2.1
	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 12 (ADAMTS)	BB004039	2
Apoptosis	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	NM_007702	2.3
Anti-apoptosis	Insulin-like growth factor 1	BG075165	2
Cytoskeleton	Microtubule-associated protein tau	M18775	2.3
Receptor activity	Brain-specific angiogenesis inhibitor 2	BB351248	2.3
Calcium ion binding	Parvalbumin	NM_013645	2.2
	S100 protein, beta polypeptide, neural	BB316114	2.1
	Secretogranin II	NM_009129	2.1

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Table 4

IFN-γ response up-regulated genes involved in the host defense pathways

Category	# genes in category	Percentage
Class I pathway and accessory molecules	32	18%
Class II pathway and accessory molecules	13	8%
Cytokine network	24	14%
Apoptosis-related genes	17	10%
Transcription factors	10	6%
Chemokines	10	6%
GTP-binding proteins (p47 GTPase family)	10	6%
HIN-200 gene family	7	4%
Antiviral actions	7	4%
Complement and Fc receptors	6	3%
Tryptophan metabolism	5	3%
Nitric oxide and respiratory burst	2	1%
Cellular adhesion molecules	2	1%
Other immune functions	27	16%

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Table 5
Validation of microarray data using RT-PCR

Gene	Fold change[a]	Change in gene vs. change in reference[b]
IIGP	296	368
TGTP	203	252
IRG-47	69	86
IGTP	43	54
GTPI	28	35
LRG-47	15	18

Fold change was calculated using the difference between Ct values between IFN-γ treated vs. untreated astrocytes for each of the different p47 GTPases and also using actin as a reference gene and change in gene vs. change in reference calculated using the Pfaffl method (Pfaffl, 2001).

[a] Fold change=Difference in Ct between IFN-γ treated vs. untreated astrocytes.

[b] Change in gene as determined using actin as a reference using the Pfaffl method.

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Table 6

IFN-γ response genes in vesicular pathways

	Gene description	**GenBank #**	**Fold change**
Up-Regulated Genes	Sorting nexin 10	AK010399	3.4
	Synapsin II	NM_013681	3.1
	RAB19, member RAS oncogene family	BM241400	2.1
	Golgi associated, gamma adaptin ear containing, ARF binding protein 2	AK004632	2.0
	Syntaxin binding protein 1	AF326545	1.9
	Ca^{2+}-dependent activator protein for secretion 2	AF000969	1.8
	Putative vesicle-associated membrane protein 5	AK009266	1.7
	Vacuolar protein sorting 54	NM_139061	1.6
Down regulated genes	Stabilin 1	NM_138672	3.1
	Rab 6B	BB39668	2.1
	VPS10 domain receptor protein SORCS	NM_021377	1.9
	Adaptor protein complex AP-1	BF458396	1.7
	Cysteine-rich hydrophobic domain 2	BB794854	1.7
	Kinesin family 1C	BQ174514	1.5

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Table 7

IFN-γ response genes in lipid pathways

	Gene description	GenBank #	Fold change
Up regulated lipid genes	Phospholipase A1 member A	NM_134102	10
	Aldehyde dehydrogenase family 1, subfamily A1	NM_013467	3.4
	Arrestin domain containing 4	BC017528	1.9
	Apolipoprotein B editing complex 3	NM_030255	1.7
	Phospholipase A2, group IVA	NM_008869	1.6
	StAR-related lipid transfer (START) domain containing 5	BI076697	1.5
	Sterol O-acyltransferase 1	BC025091	1.5
Down regulated lipid genes	Macrophage scavenger receptor 2	BC016551	6.1
	Macrophage scavenger receptor 1	AA183642	3.1
	ATP-binding cassette, sub-family A, member 8a (Abca8a)	BC026496	1.9
	Low density lipoprotein receptor-related protein 2	BM119915	1.6
	Cytochrome P450, family 39, subfamily a, polypeptide 1 (oxysterol 7 alpha-hydroxylase)	NM_018887	1.6
	Nieman–Pick type C1	BB769209	1.5