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Interferon-triggered transcriptional cascades in the oligodendroglial lineage: a comparison of induction of MHC class II antigen between oligodendroglial progenitor cells and mature oligodendrocytes

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Abstract

Interferon- γ induces major histocompatibility complex class II (MHC-II) in proliferating oligodendroglial progenitor cells (OPC), but to a much lesser extent in mature oligodendrocytes. Interferon- β has virtually no effects on MHC-II induction even in OPC. Interferon- γ -mediated transcriptional induction of *CIITA*, a critical regulator of MHC-II induction, was 6-fold lower in mature oligodendrocytes than in OPC, and entirely dependent on promoter IV, suggesting that the transcriptional activity of promoter IV is down-regulated after differentiation. The distinct difference in MHC-II induction between interferon- γ and interferon- β is attributed to transient interferon- β -mediated activation of STAT1-IRF1 signaling compared to the sustained interferon- γ -mediated activation.

Keywords

oligodendroglial lineage; interferon; MHC; IRF; *CIITA*; DNA methylation

1. Introduction

Interferons (IFNs) play essential roles in the front line of host defense against viral infections and in immunosurveillance for malignant cells. We previously demonstrated that the type II IFN, interferon- γ (IFNG), induces cytotoxic effects on oligodendroglial progenitor cells (OPC), including slowing of the cell cycle and enhancement of apoptosis, but not on myelin protein-positive mature oligodendrocytes (MO) (Horiuchi et al., 2006). IFNG is also known to induce surface expression by mature oligodendrocytes of major histocompatibility complex class I molecules (MHC-I) but not of MHC class II molecules (MHC-II) (Suzumura et al. 1986; Calder et al. 1988; Turnley et al. 1991; Satoh et al. 1991; Massa et al. 1993; Tepavcevic

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The nucleotide sequence of rat IRF2 reported in this study has been submitted to the GenBank™/EBI Data Bank with accession no. DQ674266.

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and Blakemore, 2005). This was further supported by the *in vivo* observation that myelin basic protein (MBP)-positive oligodendrocytes in actively demyelinating multiple sclerosis (MS) lesions were immunohistochemically negative to Ia-antigen (MHC-II), whereas Ia was readily demonstrable on microglia and astrocytes (Lee and Raine 1989; Gobin et al., 2001). Calder et al. (1988) demonstrated, however, that proliferating OPC are capable of expressing Ia in response to IFNG, but that they become refractory to MHC-II induction by IFNG after terminal differentiation. They suggested that loss of MHC-II inducibility associated with oligodendroglial differentiation might reduce the possibility of autoimmunization by myelin antigens. There has been no study thus far on the molecular basis for this developmental stage-dependent capability of IFNG-induced MHC-II expression in the oligodendroglial lineage.

In general, interferon- β (IFNB), one of type I interferons, is a far less potent inducer of MHC-II than is IFNG, and inhibits IFNG-induced MHC-II expression by some types of cells (Ling et al., 1985; Inaba et al., 1986; Leeuwenberg et al. 1988; Kato et al. 1989; Ransohoff et al. 1991; Lu et al. 1995; Satoh et al. 1995; Weinstock-Guttman et al. 1995). In contrast to the deleterious effects of IFNG on MS symptoms (Panitch et al., 1987), IFNB is considered one of the best therapeutic options of relapsing remitting MS to date (Clerico et al., 2008). The basic mechanisms underlying the beneficial effects of IFNB are still under investigation (Prinz et al., 2008).

Type I and Type II IFNs recognize distinct receptors, but induce overlapping groups of interferon-stimulated genes (ISGs) stemming from their common dependence on activation of signal transducer and activator of transcription 1 (STAT1) for regulation of ISG transcription. IFNG binds to the type II IFN receptor and exclusively phosphorylates STAT1 through the receptor-associated Janus activated kinases (JAKs), whereas type I IFNs, such as IFNB, recognize the type I IFN receptor, and activate STAT1 and STAT2. Activated STAT1 not only itself functions as a transcription factor after homodimerization, but also forms another transcription complex, interferon-stimulated gene factor 3 (ISGF3), together with activated STAT2 and IRF9/ISGF3G. The specific DNA motif recognized by a phosphorylated STAT1 homodimer is known as IFNG-activated site (GAS), and distinct from the IFN-stimulated response element (ISRE) where ISGF3 binds (reviewed by Platanias 2005).

Classical MHC-II genes and some MHC-II related genes are not direct targets of STAT1-mediated transcription. The promoter regions of these genes have a conserved regulatory module which is recognized by a MHC-II enhanceosome complex consisting of the RFX DNA-binding factors, CREB, and the NF-Y factors (reviewed by Boss and Jensen 2003; van den Elsen et al., 2004). These components of the MHC-II enhanceosome are expressed ubiquitously and in an apparently unregulated manner, and thus transcription of MHC-II genes is tightly and quantitatively controlled by recruitment of another trans-activator, CIITA (Steimle et al., 1994; Masternak and Reith, 2002). Which transcriptional step is responsible for the distinct difference in MHC-II induction between IFNG and IFNB in the oligodendroglial lineage? Does IFNB inhibit IFNG-mediated MHC-II induction in the oligodendroglial lineage? These questions remain to be clarified.

Rats provide a favorable model with which to address these issues of IFN-mediated MHC-II expression in the oligodendroglial lineage. Rats are the second species in which genomic sequence of almost the entire MHC region has been determined (Hurt et al., 2004), and the molecular diversity among MHC haplotypes has been well studied (Ettinger et al., 2004; Günther and Walter 2001; Vestberg et al. 1998). The rat MHC-II region encompasses approximately 300 kb encoding at least five classical class II genes, RT1-HA, RT1-BB, RT1-BA, RT1-DB1, and RT1-DA, and four other class II genes, RT1-DOA, RT1-DMA, RT1-DMB, and RT1-DOB. Compared with a high degree of plasticity within class I regions, the genomic organization of the class II region is well-conserved between humans and rodents, clearly

indicating that rat RT1-H genes are orthologous to HLA-DP in humans, the RT1-B genes to HLA-DQ, and the RT1-D genes to HLA-DR. Moreover, using antibodies for surface markers and immunopanning, we have established highly purified oligodendroglial cultures at different developmental stages (Itoh et al., 2002). These cultures are virtually free of microglia, CNS professional antigen-presenting cells which constitutively express cell surface MHC-II, and thus enable us to exclude a major concern in interpretation of the biochemical analyses of MHC-II expression. Using these cultures, we provide a detailed analysis of the developmental stage-specific transcriptional control of the genes involved in MHC-II expression by IFNG and IFNB in the oligodendroglial lineage.

2. Materials and Methods

2.1. Reagents and Chemicals

All reagents and culture media used in this study were purchased from Sigma and Invitrogen (Carlsbad, CA), respectively, except for the following products. Rat recombinant IFNG and IFNB, human recombinant fibroblast growth factor 2 (FGF2), and human recombinant platelet-derived growth factor A homodimer (PDGF_{AA}) were from R&D systems (Minneapolis, MN); the mouse monoclonal antibodies OX-18 and OX-17 for rat MHC class I (RT1A monomorphic) and class II (RT1D monomorphic), respectively, were from AbD Serotec (Raleigh, NC). Rabbit polyclonal anti-IRF1 antibody was from Santa Cruz biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-STAT1 and rabbit polyclonal anti-STAT1 antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-human Olig2 IgG was from Immuno-Biological Laboratories, Co. Ltd. (Takasaki, Japan). Rabbit polyclonal anti-NG2 chondroitin sulfate proteoglycan antibody was from Chemicon (Temecula, CA). Rat anti-mouse MHC-II (I-A/I-E) was from eBioscience (San Diego, CA).

2.2 Purified culture of rat oligodendroglial lineage

Purified primary cultures of oligodendroglial lineage from 0 to 2-day-old Lewis rats were prepared by serial immunopanning procedures as reported in detail elsewhere (Itoh et al., 2002; Horiuchi et al. 2005). Purified oligodendroglial cultures contained a more than 90% A2B5-positive O4-negative and glial fibrillary acidic protein-negative cell population which corresponded to the developmental stage of OPC. More importantly, the OPC cultures were virtually free of microglia as determined immunocytochemically by expression of CD11b antigen. The OPC cultures were expended by up to four passages in GM medium, a 3:7 mixture (v/v) of B104 neuroblastoma-condition medium and the N1 medium (high glucose Dulbecco's modified Eagle's medium supplemented with 6 mM L-glutamine, 10 ng/ml biotin, 5 µg/ml insulin, 50 µg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, and 100 µM putrescine as final concentrations) containing 5 ng/ml bovine FGF2, 1 ng/ml human recombinant PDGF_{AA}, 100 U/ml penicillin and 100 µg/ml streptomycin.

To induce differentiation of OPC to oligodendrocytes, the culture medium was switched from the GM medium to DM (differentiation medium), a 1:1 mixture (v/v) of high glucose Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 4.5 mM L-glutamine, 10 ng/ml biotin, 12.5 µg/ml insulin, 50 µg/ml transferrin, 24 nM sodium selenite, 10 nM progesterone, and 67 µM putrescine, 0.4 µg/ml 3,5,3',5'-tetraiodothyronine, 100 U/ml penicillin and 100 µg/ml streptomycin as final concentrations. Based on the immunocytochemical data (Itoh et al., 2002), we employed these oligodendroglial cultures at 4 days in DM as cultures of mature oligodendrocytes (MO).

2.3 Mouse mixed glial culture

C57BL/6J wild-type mice and IRF1-deficient mice with the same genetic background (Matsuyama et al., 1993) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Mouse mixed glial cultures were prepared from brains of 0 to 2-day-old neonates by the same methods as described for rat cultures. These cultures were maintained in GM medium.

2.4. Immunocytochemistry

Rat OPC and MO cultured on 12-mm round coverslips were treated with IFNG, IFNB or medium alone for 48 h. Then, the cells were incubated with primary antibodies for rat RT1A (1:100) or rat RT1D (1:100) at room temperature for 30 min, washed with PBS 3 times, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 min. After 3 times of wash with PBS, cells were fixed with 4% paraformaldehyde at room temperature for 10 min, washed, and permeabilized with ice-cold absolute methanol for 10 min. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml) for 15 min.

Mouse mixed glial cultures were immunolabeled by the same methods as used for rat cells, except that rabbit anti-NG2 (1:50), rat anti-mouse MHC class II (I-A/I-E) (1:50) antibodies, and corresponding secondary antibodies were used.

2.5. Flow cytometry

OPC cultures in 60-mm dishes were treated with IFNG, IFNB or medium alone. At 48 h after treatments, the cells were washed once with 2 ml of Ca²⁺- and Mg²⁺-free HBSS. The cells were detached from the plate by 0.5 ml of 0.05% (w/v) trypsin at 37°C for 2 min, and suspended in 2 ml of GM with 625 µg/ml trypsin inhibitor. After centrifuge at 520× g for 10 min, the pellet was resuspended in 5 ml of staining buffer (0.1% BSA in PBS). Soon after cell counting, 10⁵ cells were transferred into a 5 ml tube to centrifuge, and then incubated in 0.2 ml of staining buffer with primary antibodies for rat RT1A or rat RT1D (1:100) on ice for 30 min. The cells were washed 3 times with 1 ml staining buffer and incubated in 0.2 ml staining buffer with FITC-conjugated goat anti-mouse IgG antibody (1:50) on ice for 30 min. After 3 times of wash, cells were analyzed by CyAn-ADP flow cytometer (Dako Cytometry, Carpinteria, CA).

2.6. Semiquantitative RT-PCR and Quantitative PCR

Total RNA was isolated by TRIzol-Reagents (Invitrogen) or RNeasy RNA extraction kit (Qiagen, Valencia, CA) or a combination of the both. RT reaction and semiquantitative PCR were performed as reported (Itoh et al., 2003). All sets of primers used in this study are listed in Table. Small variability of cDNA contents in an experimental group was verified by semiquantitative PCR for *GAPDH*. To confirm reproducibility of the results, each RT-PCR was repeated twice using total RNA samples from independent experiments.

Quantitative PCR (qPCR) analysis was performed by MX3005P (Stratagene, La Jolla, CA) using TaqMan[®] Assay-on-Demand[™] assay kits (assay numbers: Rn00583505_m1, Rn00561424_m1, Rn00585451_m1, Rn01427980_m1, Rn00565062_m1, Rn00709368_m1 and Rn00571654_m1 for detection of *STAT1*, *IRF1*, *CIITA*, *RT1D*, *CD74*, *CD80* and *CD86*, respectively) (Foster City, CA). For the analysis of *IRF2*, Mm00515204_m1 was used because the kit for rat was not available at the time of experiments, and because we confirmed that the target sequence is identical between rats and mice (rat *IRF2*, GenBank Accession number DQ674266). The primer and probe set for rat *IRF9* was obtained by AssayByDesign (Applied Biosystems) based on the sequence information of rat *IRF9* cDNA that we subcloned. For a concentration standard, the whole coding region of each target gene, containing the target segment of the detection kit, was inserted into the cloning vector, pCR Blunt II TOPO vector, and then the purified constructs at serially diluted concentrations from 10 pM to 1 fM per strand end were used in the same reaction. *GAPDH* cDNA levels were quantified by the same system for further standardization, and the absolute cDNA amounts were expressed as ratios to

GAPDH cDNA. Analysis of each gene was repeated at least twice using total RNA samples from independent experiments and confirmed that changes in cDNA levels among experimental groups were reproducible. To determine statistical significance at the time points indicated in the text, data were obtained from at least triplicate independent experiments.

2.7. Bisulfite sequencing

Bisulfite modification of genomic DNA was performed using CpGenome™ DNA modification kit (Millipore, Billerica, MA) following the protocol accompanied with the kit. The primer pairs used to amplify the nontemplate strand and the template strand were 5'-GGGTTGTATGTGGGTGGAAG and 5'-AAACAATCTCCTAACAACACTACCTCA, and 5'-CACTCAATCCAAACAACTTAAATTAC and 5'-GTGGTTGGGTTTTGTGTTTT, respectively. Amplified products were ligated into the pCR2.1 TOPO vector (Invitrogen) and introduced into TOP10 competent cells. More than 10 transformants were randomly selected and sequenced by automated DNA sequencing at the DNA sequencing core facility of the University of California Davis.

2.8. Immunoblots

Protein samples were prepared as described previously (Horiuchi et al., 2006). Twenty µg of protein from each sample was size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and probed with primary antibody for IRF1 (1:100), STAT1 (1:1000), or phospho-STAT1 (1:500) for 1 h. Full range recombinant Rainbow Molecular Weight Markers (Amersham Biosciences) was used as a reference for molecular sizes. Immunoreactive signals were detected by enhanced chemiluminescence according to the manufacturer's protocol (Amersham Biosciences). Equal protein loading was confirmed by subsequent probing with the mouse monoclonal antibody against GAPDH (Chemicon, Temecula, CA).

2.9. Statistical analysis

Data are presented as mean $\pm$ S.D. P values were calculated by ANOVA followed by the Bonferroni/Dunn post-hoc test.

3. Results

3.1. Surface expression of MHC in the primary cultures of the rat oligodendroglial lineage in response to interferons

Both OPC and MO in control cultures expressed no immunoreactivity for either MHC-I or MHC-II. Consistent with previous studies (Massa et al., 1993; Suzumura et al., 1986; Wong et al., 1984), surface expression of MHC-I became easily detectable in 100% of both OPC and MO after a 48 h treatment with IFNG (Fig. 1A). MHC-II expression was also induced by IFNG in our oligodendroglial cultures but to a less extent compared with MHC-I. Forty eight $\pm$ 8% (n=4) of OPC became positive for MHC-II by IFNG, whereas 7 $\pm$ 1% (n=4) of MO (p<0.01) became positive, when determined by direct cell counting (Figs. 1B–C). Double immunolabeling for MHC-II and Olig2 confirmed no contamination of microglial population in the purified oligodendroglial cultures, and that surface MHC-II was induced by IFNG on OPC which were identified by nuclear immunoreactivity for Olig2 (Fig. 1D). In contrast to IFNG, IFNB was much less effective in MHC expressions on the oligodendroglial lineage. MHC-II was not detectable after a 48 h treatment of both OPC and MO with IFNB, although MHC-I was weakly induced in most of OPC and MO (Fig. 1A–C). Based on these results, we focused on the molecular mechanisms underlying the two issues of MHC-II expressions on the oligodendroglial lineage in response to the interferons; 1) Developmental down-regulation

of IFNG-mediated MHC-II expression after differentiation from OPC to MO, and 2) Virtually no induction of MHC-II by IFNB in both OPC and MO compared with that by IFNG.

3.2. Transcriptional induction of MHC class II genes by IFNG and IFNB

We performed a comprehensive analysis of transcriptional induction of MHC-II genes in OPC (Fig. 2A) and MO (Fig. 2B) in response to either IFNG or IFNB. No transcript of the five classical MHC-II genes was detected in control OPC and MO cultures by semi-quantitative RT-PCR. IFNG remarkably up-regulated RT1-BB, RT1-BA, RT1-DB, and RT1-DA mRNA in OPC and MO within 12 h. RT1-HA mRNA remained undetectable in the presence of IFNG in OPC and MO. Among other MHC-II genes, RT1-DMA mRNA was expressed at detectable levels even in control cultures of OPC and MO, and induced slightly more by IFNG. On the other hand, transcriptional induction of RT1-DMB encoding the beta chain of the RT1-DM protein was controlled in the same manner as observed in the classical MHC-II genes such as RT1-D genes. The RT1-DO genes, RT1-DOA and RT1-DOB, were not detected in control cultures, and RT1-DOA mRNA became faintly detectable only in OPC after treatment with IFNG. These results clearly demonstrated that MHC-II genes, particularly classical class II genes, were less induced by IFNG in MO than in OPC. In contrast, IFNB had much less effects on transcriptional induction of MHC-II genes than IFNG. IFNB faintly induced RT1-DA, RT1-BA, and RT1-DMB mRNAs in OPC, and only RT1-DA and RT1-BA at hardly detectable levels in MO. Moreover, induction of these genes was reduced at 24 h even in the continuous presence of IFNB, indicating that the effect of IFNB on transcriptional induction of MHC-II genes was transient. We then examined upstream molecular events underlying transcriptional induction of MHC-II genes by the interferons in the oligodendroglial lineage.

3.3. Transcriptional induction of interferon regulatory factors (IRFs) by IFNG and IFNB in the oligodendroglial lineage

Some interferon regulatory factors (IRFs) are immediate targets of JAK/STAT signaling, and directly involved in subsequent induction of various ISGs as transcription factors (reviewed by Taniguchi et al., 2001). We investigated the time-course of transcriptional induction of *IRF1*, *IRF2*, and *IRF9/ISGF3G* in OPC and MO in response to IFNG or IFNB by qPCR (Fig. 3). As reported in our previous study (Horiuchi et al., 2006), IFNG elicited a more than 70-fold sustained elevation of *IRF1* mRNA from basal levels in both OPC and MO within 60 min. The steady-state levels of IFNG-induced *IRF1* mRNA were even slightly higher in MO than in OPC, while the basal levels were 15-fold lower in MO than in OPC. *IRF2* is known to be inducible by IFNG (Cha and Deisseroth, 1994), and to function as a negative regulator of ISG expression by competing for the same IRF-binding element (IRF-E) with *IRF1* (Harada et al., 1989) and/or *ISGF3* (Hida et al., 2000). In both OPC and MO, however, *IRF2* mRNA was constitutively expressed at similar levels and not up-regulated by IFNG. *IRF9/ISGF3G*, the DNA binding subunit of *ISGF3*, was also up-regulated by the type II interferon IFNG toward approximately 10-fold higher sustained levels than basal levels in both OPC and MO. These results indicated that transcriptional induction of *IRF1*, *IRF2* and *IRF9* by IFNG could not explain the reduced induction of MHC-II by IFNG after differentiation.

In contrast to sustained elevation of *IRF1* mRNA by IFNG, IFNB induced only a transient up-regulation of *IRF1* mRNA. Within 3 h after addition of IFNB, *IRF1* mRNA levels were up-regulated, reaching a peak that was slightly lower than the plateau level induced by IFNG. Thereafter, however, *IRF1* mRNA levels in OPC and MO decreased even in the continuous presence of IFNB, falling to less than one tenth of the sustained levels induced by IFNG at 24 h (Fig. 3). On the other hand, IFNB induced sustained elevation of *IRF9/ISGF3G* mRNA for at least 24 h, indicating intact and sustained IFNB signaling in OPC as long as IFNB is present. IFNB did not change *IRF2* mRNA levels at both stages as well (Fig. 3).

Transcriptional induction of *STAT1* was also enhanced by both IFNG and IFNB, which could in turn increase *STAT1* to be activated by IFNs as a potential positive feedback. However, *STAT1* mRNA levels were up-regulated to similar levels within 6 h by either IFNG or IFNB, ruling out the contribution of this feedback mechanism to differing *IRF1* mRNA induction between IFNG and IFNB (Fig. 3).

Immunoblotting for IRF1 further confirmed robust and sustained induction of IRF1 by IFNG, but weak and transient induction by IFNB. It took approximately 2 to 3 h for IRF1 protein to reach a peak level. However, IRF1 protein declined thereafter when induced by IFNB (Fig. 4). Simultaneous immunoblotting for phosphorylated *STAT1* indicated that this weak and transient transcriptional up-regulation of IRF1 by IFNB was a consequence of far less activation of *STAT1* by IFNB than that by IFNG (Fig. 4). In agreement with the mRNA data, there was no difference in IFNG-induced IRF1 protein levels between OPC and MO (Fig. 4C). In addition, our results confirmed that IFNB did not inhibit induction of IRF1 by IFNG at both transcriptional and post-translational levels (Figs. 4 and 5). This ruled out the possibility that the subsequent decline of IFNB-induced *IRF1* mRNA was mediated by negative regulator(s) specifically induced by IFNB but not by IFNG. Moreover, IFNG at serially diluted concentrations from 100 ng/ml still induced a sustained elevation of *IRF1* mRNA in OPC until 24 h, suggesting that transient transcriptional induction of *IRF1* by IFNB is a result of IFNB-specific transcriptional regulation on the *IRF1* gene (Fig. 5B).

An indispensable role for IRF1 in IFNG-induced MHC-II expression in OPC was confirmed by the mixed glial cultures from IRF1-deficient mice. A considerable number of mouse OPC could be identified in the mixed glial cultures by surface immunoreactivity for NG2 chondroitin sulfate proteoglycan, and they were negative for surface MHC-II in the absence of IFNG. After addition of mouse IFNG, MHC-II was expressed on NG2-positive OPC in the wild-type cultures, whereas MHC-II immunoreactivity remained undetectable in IRF1-deficient NG2-positive OPC (Fig. 6).

3.4. Transcriptional induction of *CIITA* is 6-fold less in MO than in OPC

We then investigated transcriptional induction of *CIITA* and *RT1-DA*, one of classical MHC-II genes, by either IFNG or IFNB in OPC and MO (Fig. 7). *RT1-DA* gene was selected for the qPCR analysis, because *RT1-DA* shows the lowest degree of amino-acid polymorphism in all identified allotypes among rat classical MHC-II genes (Ettinger et al., 2004; Vestberg et al. 1998), and was clearly up-regulated by the IFNs based on our semi-quantitative RT-PCR. As shown in Fig. 7, basal levels of *CIITA* mRNA were extremely low in control cultures of both OPC and MO. *CIITA* mRNA was increased rapidly from 1 to 6 h after the addition of IFNG, which was delayed by approximately 2 h from the up-regulation of *IRF1* and *IRF9* by IFNG. *CIITA* mRNA was maintained at elevated levels as long as IFNG was present. Interestingly, these steady-state levels were approximately 6-fold less in MO than in OPC. *RT1-DA* mRNA levels were also close to or below the detectable limit by the qPCR analysis in both control cultures. This again confirmed the absence of microglia which constitutively express MHC-II in our highly purified oligodendroglial cultures. Following induction of *CIITA* mRNA, *RT1-DA* mRNA was dramatically up-regulated to more than 1000-fold levels from 3 to 12 h. In an excellent correlation with 6-fold lower induction of *CIITA* mRNA in MO than in OPC, the plateau levels of *RT1-DA* mRNA by IFNG were 4-fold lower in MO than in OPC.

On the other hand, IFNB only transiently up-regulated *CIITA* mRNA in the same kinetic pattern as observed in the induction of *IRF1* mRNA by IFNB. After reaching peak levels at 6 h, *CIITA* mRNA was rapidly reduced along with time. At 24 h after the addition of IFNB, the mRNA levels were 100-fold less than those maintained by IFNG in OPC, and returned to the level close to the detection limit in MO. In contrast to the rapid reduction of *CIITA* mRNA,

RT1-DA mRNA levels were maintained at similar levels from 6 to 24 h. However, these levels were more than 20-fold lower than those maintained by IFNG.

The transcriptional induction of MHC-associated invariant chain (CD74/Ii), an essential molecule for conventional MHC-II biosynthesis and maturation and antigenic peptide loading, in response to IFNG or IFNB was nearly identical to the induction of *RT1-DA* by IFNG or IFNB, respectively, except that the basal expression levels were slightly increased after differentiation from OPC to MO (Fig. 7).

3.5. Expression of co-stimulatory molecules are independent of the interferons in the oligodendroglial lineage

Members of the B7 family of costimulatory molecules are up-regulated by exposure to IFNG in mouse astrocytes (Soos et al., 1999; Girvin et al., 2002), and thus play essential roles in development of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Chang et al., 1999). qPCR revealed that *CD80* (*B7-1*) mRNA was constitutively expressed in OPC at levels similar to the basal levels in the spleen. After differentiation from OPC to MO, basal levels of *CD80* mRNA were 10-fold down-regulated. Neither IFNG nor IFNB up-regulated *CD80* mRNA levels in OPC and MO. In contrast to *CD80*, *CD86* (*B7-2*) transcripts were virtually undetectable in both OPC and MO regardless of the presence of the interferons (Fig. 8). These results clearly indicate that the B7 family of costimulatory molecules is not controlled at the transcriptional level by the interferons in the oligodendroglial lineage.

3.6. The methylation pattern of promoter IV of the *CIITA* gene demonstrates a modest change after differentiation from OPC to MO

IFNG-mediated transcriptional induction of *CIITA* was down-regulated after differentiation from OPC to MO. Transcription of *CIITA* is controlled by at least three distinct and independent promoter regions, spread over a large (>12 and >9 kb in human and rat, respectively) genomic region, each transcribing a unique first exon. The results of the qPCR analysis of *CIITA*, which detected the exon 12/13 junction, indicated that all promoters of *CIITA* were silent in the oligodendroglial lineage in the absence of IFNG. Among these promoters, promoter IV (pIV hereafter) regulates IFNG-inducible expression of *CIITA* in most nucleated cells (Muhlethaler-Mottet et al., 1997). Semi-quantitative RT-PCR specific for the exon 1/2 boundary of each unique transcript confirmed that pIV was solely responsible for IFNG-mediated expression of *CIITA* in the oligodendroglial lineage as well (data not shown). In some types of cells, for example placental trophoblasts, hypermethylation of CpG dinucleotides across the broad region of pIV is responsible for silencing of the pIV activity (Morris et al., 2000, 2002). To examine this regulatory mechanism, we compared the methylation pattern of pIV between OPC and MO by bisulfite sequencing. The results demonstrated a modest change in the methylation pattern after differentiation, but failed to prove broad hypermethylation of pIV in MO compared to OPC (Fig. 9). In particular, the CpG dinucleotide numbered as 2 in Fig. 9, which is located in the E-box and conserved between human and rodents, was not more frequently methylated in MO than in OPC.

4. Discussion

We have addressed molecular mechanisms underlying the following two issues in this study; 1) Developmental reduction in IFNG-mediated expression of MHC-II genes after differentiation from OPC to MO, and 2) Much less effect of IFNB on MHC-II induction than that of IFNG in the oligodendroglial lineage. The two issues are discussed separately.

4.1. Developmental reduction in IFNG-mediated expression of MHC-II genes

In accordance with the early observation by Calder et al. (1988) on O-2A cells, we first confirmed that, in our highly purified primary oligodendroglial cultures, OPC can express surface MHC-II in response to IFNG without interaction of other cell types, and that this IFNG-induced MHC-II expression is reduced after terminal differentiation to MO. In both OPC and MO, *IRF1* mRNA was upregulated within 1 h after the addition of IFNG, and maintained at 70-fold or more higher than basal levels as long as IFNG was present in the cultures. Thus, as far as could be determined by induction of *IRF1* mRNA and protein, both OPC and MO have substantially equal functional JAK/STAT pathways, a conclusion consistent with our previous study (Horiuchi et al., 2006).

In contrast to similar transcriptional induction of *IRF* genes in OPC and MO, our results demonstrated that IFNG-mediated inductions of *RT1DA* and *CD74/Ii* were significantly reduced at the transcriptional levels after differentiation from OPC to MO. In the oligodendroglial lineage, basal levels of *CIITA* mRNA were undetectable by qPCR analysis, and IFNG dramatically up-regulated *CIITA* mRNA in a time frame that preceded induction of MHC-II mRNA. Significantly, induction levels of *CIITA* mRNA were 6-fold less in MO than in OPC, in a good correlation with the reduced induction of *RT1DA* and *CD74/Ii* after differentiation. Therefore, it is reasonable to conclude that the lesser transcriptional induction of *CIITA* by IFNG in MO than in OPC is largely attributable to lower surface expression of MHC-II in MO than in OPC.

Among at least three distinct promoters of the *CIITA* gene, pIV is solely responsible for IFNG-mediated expression of *CIITA* in the oligodendroglial lineage. Three cis-acting elements situated within 200 bases from the transcription initiation site, GAS, E box, and IRF-E, are essential for activation of *CIITA* pIV by IFNG (Muhlethaler-Mottet et al., 1998; O'Keefe et al., 2001). Morris et al. (2002) pointed out that, although activated STAT1 bound to the GAS within 15 min of IFNG signaling, pIV was not activated until IRF1 protein was accumulated above a concentration sufficient to occupy its IRF-E. Our results in the oligodendroglial lineage support their kinetic model, because induction of *CIITA* mRNA was delayed by at least 1 h after *IRF1* mRNA was fully induced. In spite of the similar induction levels and kinetics of *IRF1* mRNA between OPC and MO, however, MO demonstrated 6-fold less induction of *CIITA* mRNA in response to IFNG. Resistance to IFNG-inducible MHC-II expression has also been observed in peritoneal and alveolar macrophages from neonates, trophoblasts in placenta, and tumor cells of various origins (Lee et al. 2001; Croce et al. 2003). Particularly in the latter two cell types, pIV activity is inhibited or silenced by DNA hypermethylation at CpG dinucleotides across the broad region of pIV as an epigenetic modification, providing them a privilege to escape from immune surveillance even in the presence of IFNG (Morris et al., 2000, 2002; Croce et al. 2003). However, the results of bisulfite sequencing did not fully support the contribution of this repressive epigenetic modification to the reduced pIV transcriptional activity in MO, suggesting that other epigenetic mechanisms such as histone deacetylation and/or methylation might regulate transcriptional activity of pIV during differentiation of the oligodendroglial lineage (Wright and Ting 2006). Protein kinase C activity is also known to regulate *CIITA* expression by selectively modulating the transcriptional activity of IRF1 in some tumor cells (Giroux et al. 2003). Although future experiments are required to clarify which epigenetic modifications contribute to the reduced pIV transcriptional activity in MO, the down-regulation of IFNG-mediated induction of *CIITA* mRNA may be a key physiological mechanism to protect myelinating oligodendrocytes from immune attack or loss.

4.2. IFNB has much less effect than IFNG on MHC-II expression by the oligodendroglial lineage

Our kinetic analysis of the IFNB-induced transcriptional cascade indicated that poor MHC-II expression by IFNB is largely a consequence of the transient and weak transcriptional induction of IRF1 by IFNB. The initial phase of IFNB-induced transcription of ISGs is mediated by ISGF3 (STAT1 +STAT2 +IRF9/ISGF3G) and STAT1 homodimers (Platanias 2005). ISGF3 recognizes ISRE, the core sequence of which is a tandem repeat of the GAAANN motif, almost indistinguishable from that of IRF-E, but distinct from the palindromic core sequence of GAS recognized by a STAT1 homodimer. The promoter region of the *IRF1* gene contains one typical GAS but neither typical ISRE nor IRF-E. Pine et al. (1994) and Park et al. (2000) demonstrated that another type I IFN, interferon- α , induced *IRF1* mRNA by binding of phosphorylated STAT1 to the single GAS in the *IRF1* promoter region. In contrast, the DNA binding subunit of ISGF3, IRF9/ISGF3G, had little effect on the expression of *IRF1* (Cha and Deisseroth, 1994). Therefore, the initial up-regulation of *IRF1* mRNA by IFNB is likely to be mediated exclusively by activated STAT1 homodimers through the GAS. Activated STAT1 was almost undetectable in IFNB-treated OPC by our immunoblots (Fig 4), but a small amount could be present in the initial phase of IFNB signaling, accounting for the weak initial induction of IRF1. However, the formation of STAT1 homodimers may be further reduced with time, because activated STAT1 is used for the formation of ISGF3 along with IFNB-mediated activation of STAT2 and de novo synthesis of IRF9/ISGF3G, thus resulting in the rapid decline of IRF1 transcripts. Moreover, both STAT1 mRNA and protein were further up-regulated by both IFNG and IFNB, as a potential positive feedback of STAT1 signaling (Fig. 3 and Fig. 4). Although IFNG- and IFNB-mediated up-regulations of STAT1 were almost equivalent at mRNA levels, total STAT1 protein levels were apparently lower in OPC treated with IFNB than those with IFNG from 6 to 24 h (compare Fig. 3 and Fig. 4). This difference is presumably due to faster degradation of nonphosphorylated STAT1 located outside of the nucleus compared to phosphorylated STAT1 translocated into the nucleus. Collectively, our results indicate that GAS-driven IRF1 transcription plays a key role in differential MHC-II expression between IFNG and IFNB in the oligodendroglial lineage.

IFNB has an antagonistic effect on the IFNG-induced expression of MHC-II in macrophages and astrocytes (Ling et al. 1985; Inaba et al. 1986; Ransohoff et al. 1991; Lu et al. 1995; Satoh et al. 1995). In purified oligodendroglial progenitors, however, IFNB had no inhibitory effects on IFNG-induced *IRF1* mRNA and protein levels (Fig. 4 and Fig. 5A) and *RTIDA* mRNA level (data not shown).

4.3. Implications of IFNG-mediated MHC-II expression on OPC in neuroinflammation

The biological significance of MHC-II inducibility by IFNG in OPC *in vivo* remains an open question. Although there might be differences in MHC-II induction between the neonatal OPC and the adult OPC, OPC have not been subjected to immunohistochemical scrutiny for MHC-II expression in various types of CNS inflammation in rodents and humans. Moreover, IFNG-mediated MHC-II induction in OPC, even at lower levels than the detection limits by immunohistochemistry, might be involved in CNS autoimmunity, remyelination (Arnett et al., 2003) and allograft rejection (Tepavcevic and Blakemore, 2005). These issues need to be addressed in future *in vivo* studies.

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Figure 1.

Surface expression of major histocompatibility complex on cells of rat oligodendroglial lineage in response to IFNG or IFNB. Surface immunoreactivities for RT1A and RT1D in OPC (**A**) and MO (**B**) were examined by immunocytochemistry at 48 h after treatment with IFNG, IFNB or medium alone (control). RT1A (class I, green) was absent in control cultures, and more strongly induced by IFNG than by IFNB. RT1D (class II, green) was only induced by IFNG in OPC, and, to a much less extent, in MO. Nuclei were visualized with DAPI (blue). Scale bar: 50 μ m. **C**: Flow cytometry confirmed that RT1D-positive population of OPC at 48 h after treatment with IFNG was 50% (R1), whereas less than 1% of the population was positive in IFNB-treated cultures. **D**: Olig2-positive OPC did express RT1D in the presence of IFNG.

OPC cultures were treated with IFNG (100 ng/ml) or medium alone for 48 h, and immunolabeled for Olig2 (red) and RT1D (green). Nearly all cells in both OPC cultures were positive for nuclear Olig2, and some Olig2-positive cells were surface positive for RT1D after addition of IFNG (arrowheads). Nuclei were visualized with DAPI (blue). Scale bar: 50 μ m.

Figure 2.

Expression profiles of rat MHC-II genes in oligodendroglial progenitor cells (A) and mature oligodendrocytes (B) in response to IFNG and IFNB by semi-quantitative RT-PCR. Total RNA was collected from the cells treated with IFNG (100 ng/ml) or IFNB (1000 U/ml) for 12 and 24 hours. Control cultures (Control) were treated with the culture medium alone for 0, 12, and 24 hours. Samples from rat spleen were used as positive controls (pC). Equal loading of the samples was confirmed by the reaction for GAPDH as shown at the bottom.

Figure 3.

Quantitative analysis of induction of *IRF1*, *IRF2*, *IRF9/ISGF3G*, and *STAT1* mRNA in OPC (left column, OPC) and MO (right column) after the addition of IFNG (100 ng/ml, closed circle), IFNB (1000 U/ml, closed triangle), or medium alone (Control, open circle). Note that the data are plotted as ratios to copy numbers of *GAPDH* cDNA on a logarithmic scale. At time 0, data from controls are only shown. Statistical significance of transcriptional up-regulation of *IRF1* and *IRF9* at 24 h after the treatments was examined with at least 3 independent sets of experiments (far right graph). ** indicates $P < 0.01$.

Figure 4.

A and B: Time-course of phosphorylation of STAT1, and up-regulation of STAT1 and IRF1 proteins in OPC in response to IFNG (**A**) or IFNB (**B**). Protein aliquots (20 μ g) from OPC incubated with IFNG (100 ng/ml), IFNB (1000 U/ml) or both (far right lanes in A and B) for the indicated time-periods were immunoblotted for phosphorylated STAT1, total STAT1, and IRF1. The two arrowheads in the phosphorylated STAT1 and total STAT1 panels indicate the two alternatively spliced forms, STAT1 α (91 kDa, top) and STAT1 β (84 kDa, bottom). Note that the faint signals of IRF1 in IFNB-treated OPCs from 1 h to 24 h with a peak induction at 3 h. **C:** IRF1 induction by the interferons in comparison between OPC and MO. Protein aliquots (20 μ g) from OPC and MO treated with IFNG (G, 100 ng/ml), IFNB (B, 1000 U/ml) or the

culture medium alone (C) for 24 h were immunoblotted for IRF1. IRF1 protein was robustly induced by IFNG in both OPC and MO, but only faintly by IFNB in OPC. The subsequent immunoblot for GAPDH is shown for equal protein loading.

Figure 5.

A: IFNB did not inhibit IFNG-mediated induction of *IRF1* mRNA. OPC cultures were treated with IFNG (100 ng/ml, closed circle), IFNB (1000 U/ml, closed triangle), or both (open square) for indicated durations. The value from the sample at time 0 is plotted as control (Control, open circle), because fluctuation of *IRF1* mRNA levels in control cultures within 24 h was small as shown in Figure 3. **B:** Time course of *IRF1* mRNA induction by IFNG at serially diluted concentrations (1, 10, 100 ng/ml) in OPC. Control is shown as in A. Data are plotted as ratios to copy numbers of *GAPDH* cDNA on a logarithmic scale.

Figure 6.

IRF1 was indispensable for IFNG-mediated MHC-II induction in OPC. Mixed glial cultures from wild-type (**A**, **C**) and IRF1-deficient mice (**B**, **D**) were incubated with medium alone or mouse IFNG (100 ng/ml) for 24 h, and immunolabeled for NG2 (red) and MHC-II (green). Nuclei were visualized with DAPI (blue). In the presence of IFNG, NG2-positive OPC became surface positive for MHC-II in the wild-type cultures (arrowheads in **C**), whereas MHC-II immunoreactivity remained undetectable in IRF1-deficient NG2-positive OPC (**D**). In contrast, some NG2-negative glial cells were constitutively surface positive for MHC-II even in the control cultures from both wild-type and IRF1-deficient mice (arrows in **A** and **B**, respectively). Scale bar: 50 μ m.

Figure 7.

Quantitative analysis of induction of *CIITA*, *RT1-DA*, and *CD74* mRNA in OPC (left column, OPC) and MO (right column) after the addition of IFNG (100 ng/ml, closed circle), IFNB (1000 U/ml, closed triangle), or medium alone (Control, open circle). The data are plotted as ratios to copy numbers of *GAPDH* cDNA on a logarithmic scale. At time 0, data from controls are only shown. Statistical significance of transcriptional up-regulation of *CIITA* and *RT1-DA* at 24 h after the treatment with IFNG was examined with at least 3 independent sets of experiments (far right graph). ** and * indicate $P < 0.01$ and $P < 0.05$, respectively.

Figure 8.

Quantitative analysis of *CD80/B7-1* and *CD86/B7-2* mRNA in OPC (left column, OPC) and MO (right column) after the addition of IFNG (100 ng/ml, closed circle), IFNB (1000 U/ml, closed triangle), or medium alone (Control, open circle). Basal levels of *CD80/B7-1* and *CD86/B7-2* mRNA in the samples from spleen are shown as positive controls in the panel for MO (open triangle, one symbol represents one sample). The data are plotted as ratios to copy numbers of *GAPDH* cDNA on a logarithmic scale.

Figure 9.

Methylation patterns of CpG dinucleotides in the core region of CIITA promoter IV (pIV) in OPC and MO. Genomic organization of rat CIITA pIV is shown in the upper row. Key motifs recognized by transcription factors are indicated as hatched boxes, and the nucleotide number starts from the adenine of the ATG codon in the exon 1 transcribed by promoter III. Each CpG is numbered as indicated in the schematic genomic map. Bisulfite modification of the genomic DNA from OPC and MO was performed. The nontemplate and template strands of pIV were PCR-amplified, and cloned into the cloning vector. More than 10 clones were sequenced. The percentage of each methylated CpG in the sequenced clones was calculated and presented in bar graphs. Only CpG number 2 (in bold) is conserved between human and rodents.

Table

Sequences of primers for semi-quantitative RT-PCR.

Gene	Forward primer	Reverse primer
RTIDA	CCTGTCTAGGGTGTCTTTTGC	TCACGAGAACAAAGCAGGAAA
RTIDBI	CCGCTGCGGTTTCAGTCT	AGTGAAGCCGAGGAACCACTC
RTIBA	TGGGCTTTGGAAGGTAAAGAA	GCTTGGGAGAGAAGGACTTGA
RTIBB	CTGTGGTTGTGCTGATGGTG	TCACTGTAGGAGCCCTGCTG
RTIHA	TCTCAGCTCTTACCGGCTTTC	TTCACCTTCAGTGAGCTGCAT
RTIDMA	TCCTTGAGCCACTTGAAGGAT	GCCTATGCTAACCTGCTCAC
RTIDMB	CTTCAGCCCCAGAGACAGTG	CTCTGCTTTCACAGCCATCAC
RTIDOA	GAGCTGCAGGACTGGAAGAAG	TGGCACCAAGTGAGAAGAGAG
RTIDOB	GACCCAAGGATGAGGTCTTTG	TCAAACCTCCAAGCAATGGTG
GAPDH	ATTGTCAGCAATGCATCCTGCA	AGACAACCTGGTCCTCAGTGTA