Monocytes and CD4+ T cells contribution to the under-expression of NR4A2 and TNFAIP3 genes in patients with multiple sclerosis

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Abstract

We recently found a gene signature for multiple sclerosis (MS) that reverted to normal during pregnancy in MS patients and included NR4A2 and TNFAIP3, key molecules in anti-inflammatory processes. Here we focus on the expression levels of these two genes in monocytes and CD4 + T cells from healthy controls and treatment-naïve RRMS patients. Our findings show that monocytes play a key role in the dysregulated anti-inflammatory response, being the expression of both genes down-regulated in these cells in RRMS patients with respect to healthy individuals. CD4 + T cells seem to have only a marginal part, because we can observe only a slight down-regulation in NR4A2.

Keywords

TNFAIP3; NR4A2; Multiple sclerosis; Monocytes; CD4 + T cells

1. Introduction

In a previous study we showed that gene expression of seven genes was normalized during pregnancy (Gilli et al., 2010). In the present work we focused on two out of seven genes, namely nuclear receptor subfamily 4, group A, member 2 (NR4A2) and tumor necrosis factor, α-induced protein 3 (TNFAIP3), because they play a key role in the negative regulation of inflammation (Wertz et al., 2004 and Saijo et al., 2009), and have been already involved in the pathogenesis of several autoimmune and neurodegenerative disorders (Plenge et al., 2007, Musone et al., 2008, Dieudé et al., 2010 and Ma and Malynn, 2013). Furthermore, recently other groups have demonstrated their association with multiple sclerosis (Achiron et al., 2004, Achiron et al., 2010,

Mandel et al., 2004, Satoh et al., 2005 and International Multiple Sclerosis Genetics Consortium, 2013).

NR4A2 belongs to the NR4A subfamily of orphan nuclear receptors. NR4As are early response genes whose expression is induced by various stimuli including cyclic AMP, growth factors, inflammatory signals, and hormones (Maxwell and Muscat, 2006). In recent years the role of NR4A2 in MS has been investigated, obtaining conflicting results. On one hand, it has been demonstrated that NR4A2 is involved in controlling the transcription of NF-κB target genes (Saijo et al., 2009), and in the development of Treg cells (Sekiya et al., 2011 and Sekiya et al., 2013); its low expression has been correlated with MS (Achiron et al., 2004, Achiron et al., 2010 and Mandel et al., 2004). On the other hand, it has been shown to have a role in the differentiation of Th17 cells (Raveney et al., 2013) and to be overexpressed in a population of Japanese MS patients (Satoh et al., 2005).

TNFAIP3 plays a key role in the restriction of NF-κB signaling (Wertz et al., 2004), and is also involved in the promotion of cell-survival signals (Lee et al., 2000). Single nucleotide polymorphisms (SNPs) in the TNFAIP3 gene have been linked with susceptibility to different human autoimmune diseases, such as rheumatoid arthritis (Plenge et al., 2007), systemic lupus erythematosus (Musone et al., 2008), systemic sclerosis (Dieudé et al., 2010), and only recently to MS (International Multiple Sclerosis Genetics Consortium, 2013). Besides our abovementioned work (Gilli et al., 2010), the down-regulation of TNFAIP3 expression in MS patients has been highlighted by Mandel and colleagues (Mandel et al., 2004).

To investigate the function of these two genes, we aimed at identifying particular cell subpopulations having a pivotal role in the unbalanced expression of NR4A2 and TNFAIP3. To this purpose, we performed real-time PCR experiments on monocytes and CD4 + T cells obtained from a new cohort of healthy controls and MS patients of Caucasian origin.

Different studies have highlighted an important role of monocytes in MS pathogenesis. In fact, circulating monocytes from MS patients are present in active brain lesions, and upon stimulation during acute disease relapses, they express more pro-inflammatory cytokines (Bustamante et al., 2013).

Furthermore, in the presence of inflammatory diseases of the central nervous system, bone marrow-derived monocytes migrate into the brain and differentiate into microglia, the macrophages of the central nervous system (Chan et al., 2007).

CD4 + T cells have a central part in the regulation of the adaptive immune system, and they also interact with the innate immune system. On the basis of various conditions, such as the presence of specific cytokines and co-stimulator factors, CD4 + T cells are able to differentiate into several subpopulations, possessing peculiar functions (Korn et al., 2009 and Vahedi et al., 2013). In addition to the well-established polarization Th1/Th2, evidences have demonstrated the ability of CD4 + T cells to differentiate into Th17 and Treg cells, which are both implicated in the pathogenesis of MS (Sakaguchi et al., 2008).

2. Materials and methods

2.1. Study subjects

Twenty-four patients with relapsing-remitting MS (RRMS) according to the McDonald criteria (McDonald et al., 2001) (median age: 39 ± 10; median disease duration: 21 months ± 78.9) and eighteen healthy volunteers (median age: 33.5 ± 9.6) were enrolled for the present study, after giving their written consent. All blood samples from MS patients were taken before the initiation of treatment with any disease-modifying therapy. The study was approved by the IRB of San Luigi University Hospital.

2.2. Cell separation

Peripheral blood mononuclear cells were isolated from 15 ml of peripheral blood by density gradient centrifugation with Histopaque-1077 (Sigma Aldrich, St. Louis, USA). CD4 + T cells were isolated by direct magnetic labeling using the CD4 MicroBeads kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The isolation of monocytes (CD14 +) was obtained using the Monocyte Isolation Kit II, an indirect magnetic labeling system (Miltenyi).

2.3. Real time PCR

A phenol/chloroform extraction was performed to isolate total RNA from monocytes and CD4 + T cells. Then RNA was reverse transcribed with the High Capacity Reverse Transcription Kit (Lifetechnologies, Monza, Italy) at a final concentration of 5 ng/µL. cDNA was used as a template for a real-time PCR reaction analysis of TNFAIP3 and NR4A2 genes using Taqman assays (Lifetechnologies). Relative expression levels of the two genes were calculated by normalizing to glyceraldehyde-phosphate-dehydrogenase.

2.4. Statistical analysis

Data were analyzed with GraphPad Prism, version 5.0 (GraphPad Software Inc., La Jolla, USA). Normality was tested using the Shapiro–Wilk normality test. Differences in gene expression were tested with a nonparametric 2-tailed Mann–Whitney test; p values ≤ 0.05 were considered significant.

3. Results

3.1. Expression of NR4A2 and TNFAIP3 genes in monocytes and CD4 + T cells

3.1.1. CD4 + T cell expression of NR4A2 and TNFAIP3

Comparing NR4A2 gene expression between MS patients and healthy subjects in CD4 + T cells, we observed lower levels in MS patients than in healthy controls ($p = 0.0489$) (Fig. 1A).
TNFAIP3 expression in CD4 + T cells was higher in healthy volunteers than in MS patients, approaching statistical significance ($p = 0.0519$) (Fig. 1B).

3.1.2. Monocyte expression of NR4A2 and TNFAIP3

Evaluating NR4A2 gene expression between healthy volunteers and MS patients in monocytes, lower levels were found in RRMS patients than healthy individuals ($p = 0.0083$) (Fig. 1C).
TNFAIP3 gene expression was also lower in monocytes of RRMS patients than in healthy volunteers ($p = 0.0214$) (Fig. 1D).

3.1.3. Level of expression of NR4A2 in CD4 + T cells and monocytes

Comparing NR4A2 gene expression between CD4 + T cells and monocytes in both healthy volunteers and MS patients, no differences were found (Fig. 2A).

3.1.4. Level of expression of TNFAIP3 in CD4 + T cells and monocytes

TNFAIP3 gene expression was higher in CD4 + T cells with respect to monocytes in both MS patients ($p = 0.0031$) and healthy volunteers ($p = 0.0598$) (Fig. 2B).

3.2. Correlation with clinic

There was no difference in expression levels of both NR4A2 and TNFAIP3 between MS patients with high (≥ 1 relapse or an increase of EDSS ≥ 0.5 or both the conditions 24 months after initiation of the therapy) and low (no relapse or increase of EDSS 24 months after initiation of the treatment) disease activity (data not shown).

4. Discussion

In a previous study we demonstrated that NR4A2 and TNFAIP3 levels were down-regulated in peripheral blood of MS patients compared with healthy individuals (Gilli et al., 2010). To investigate which cell subpopulation is mainly involved in this gene expression deregulation, we focused on monocytes and CD4 + T cells, which have a complex role in inflammation. In the present study we observe that monocytes may play a pivotal role in the unbalanced expression of NR4A2 and TNFAIP3 between MS patients and healthy controls. In fact, although both genes have diminished levels in MS patients with respect to healthy volunteers in both CD4 + T cells and monocytes, in the latter the down-regulation results more pronounced.

In the past years different groups have studied the role of NR4A2 in inflammation and autoimmune diseases and its function appears to be controversial.

A reduction in NR4A2 expression leads to exaggerated inflammatory responses in microglia, resulting in an amplified response by astrocytes. This anti-inflammatory activity is mediated by the recruitment of the CoREST complex by NR4A2. The Nurr1/CoREST pathway restores the transcription of NF-κB target genes to a basal state, limiting inflammatory responses of microglia and astrocytes in Parkinson's disease (Saijo et al., 2009).

A recent study showed that NR4A2 and other neuroprotective molecules can “calm” microglia after the intervention of mesenchymal stem cells and the release of CX3CL1 (Giunti et al., 2012). Furthermore, low levels of NR4A2 transcript have been correlated with having or developing multiple sclerosis (Achiron et al., 2004, Achiron et al., 2010 and Mandel et al., 2004).

NR4A2 was also seen to direct the development of both Treg (Sekiya et al., 2013) and Th17 (Raveney et al., 2013) cells, resulting respectively in the protection and exacerbation of autoimmune diseases.

Finally, in a cDNA microarray analysis performed on CD3 + T cells isolated from Japanese MS patients and healthy controls, 173 genes were found differently expressed between the two groups. One of these genes was NR4A2, which was up-regulated in MS T cells (Satoh et al., 2005). These discrepancies can be due to different techniques and modality of selection of cell subpopulations, as we evaluated CD4 + T cells that are only a part of CD3 + T cells studied by Satoh and colleagues; moreover differences between a “Western” and an “Asian” type of multiple sclerosis are well recognized (Kira et al., 1996).

Concerning the TNFAIP3 gene, less is known about its relation to MS, although its implication in several autoimmune diseases has been well established (Ma and Malynn, 2013). In 2004, Mandel and colleagues performed a microarray analysis, in which TNFAIP3 was seen to be a part of a common autoimmune gene expression signature that differentiated MS and systemic lupus erythematosus patients from healthy subjects (Mandel et al., 2004). Furthermore, the OLIG/TNFAIP3 gene region has been correlated with increased levels of CXCL13, a chemokine expressed in active MS lesions which is up-regulated in the CSF of MS patients (Lindén et al., 2013). More recently, SNPs in TNFAIP3 gene have been correlated with susceptibility to MS (International Multiple Sclerosis Genetics Consortium, 2013).

In conclusion, our results suggest a protective and anti-inflammatory role of NR4A2 and TNFAIP3, both being under-regulated in monocytes and CD4 + T cells of MS patients. The diminished expression of NR4A2 in patients' monocytes seems to be consistent with the hypothesis that its reduction may lead to an overstated inflammation in microglia, in this case predisposing and contributing to a persistent inflammation in MS (Lassmann, 2014).

Acknowledgments

We would like to thank nurses for helping in blood collection and our patients and healthy volunteers for their participation. This study was supported by grants from FISM (Italian Multiple Sclerosis Foundation): code 2010/R/7 and by Compagnia di San Paolo: code 2010/R/28.

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Figure 1. Comparison of NR4A2 (A) and TNFAIP3 (B) expression levels between MS patients (MS) and healthy controls (HC) in CD4 + T cells. Differences in expression levels of NR4A2 (C) and TNFAIP3 (D) between HC and MS in monocytes. Bars indicate the median values.

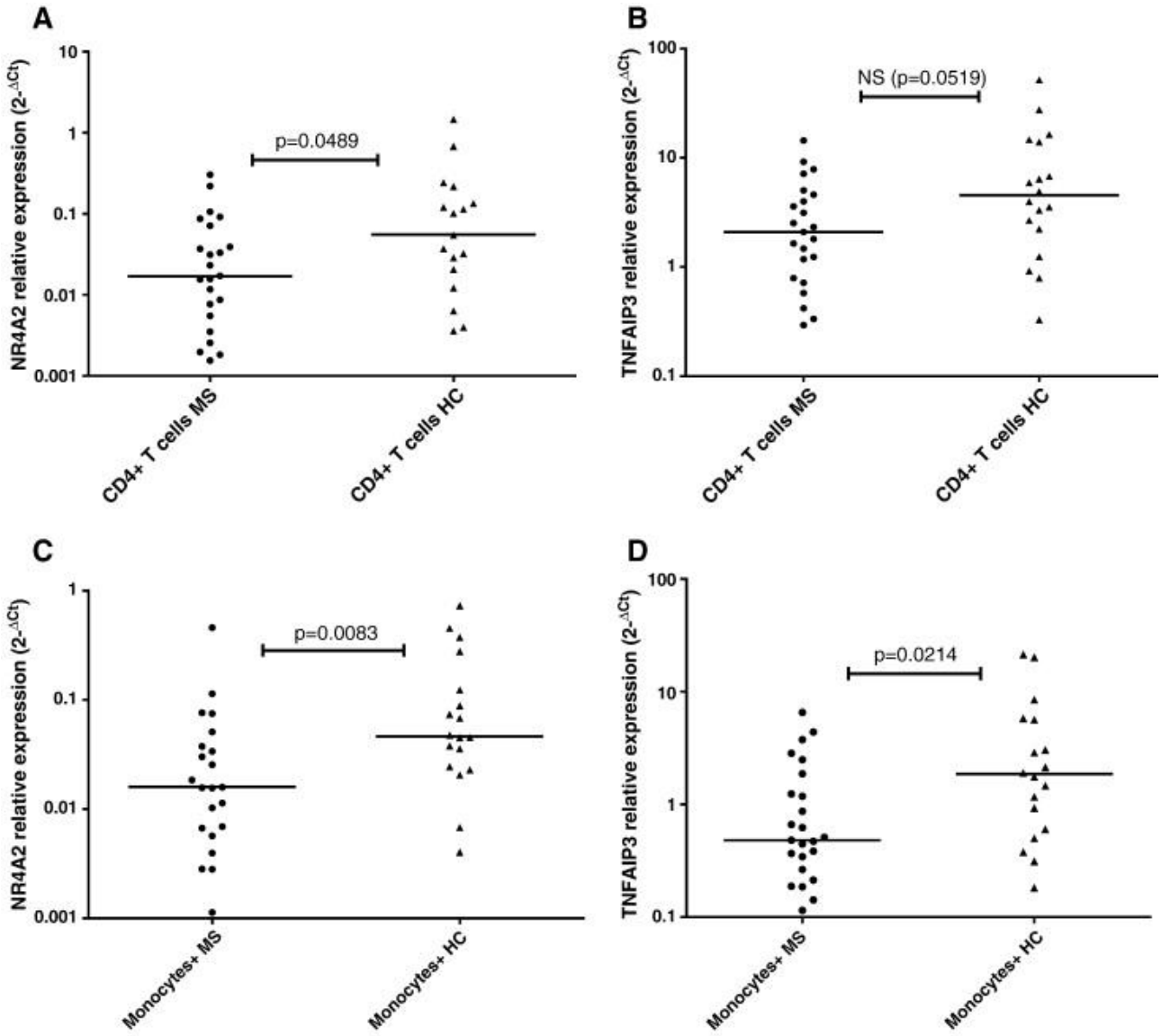

Figure 2. Expression level of NR4A2 (A) and TNFAIP3 (B) in monocytes and CD4 + T cells in both MS and HC. Bars indicate the median values.