TITLE PAGE

Title: Production of IL-27 in multiple sclerosis lesions by astrocytes and myeloid cells: modulation of local immune responses

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Main Points

Astrocytes and microglia/macrophages are sources of the elevated IL-27 in MS lesions.

Astrocytes and infiltrating T cells expressed the IL-27 receptor in MS tissues.

IL-27 induces STAT1-dependent MHC class I upregulation in human astrocytes.

Keywords: cytokine, astrocytes, microglia, T lymphocytes, neuroinflammation

ABSTRACT

The mechanisms whereby human glial cells modulate local immune responses are not fully understood. Interleukin-27 (IL-27), a pleiotropic cytokine, has been shown to dampen the severity of experimental autoimmune encephalomyelitis, but it is still unresolved whether IL-27 plays a role in the human disease multiple sclerosis (MS). We investigated if IL-27 contributes to local modulation of immune responses in the brain of MS patients. Compared to normal control brains we observed elevated expression of IL-27 subunits (EBI3 and p28) and its cognate receptor IL-27R (the gp130 and TCCR chains) within post-mortem MS brain lesions. Moreover, astrocytes (GFAP+ cells) as well as microglia and macrophages (Iba1+ cells) were important sources of IL-27. Brain-infiltrating CD4 and CD8 T lymphocytes expressed the IL-27R specific chain (TCCR) implying that these cells could respond to local IL-27 sources. In primary cultures of human astrocytes inflammatory cytokines increased IL-27 production, whereas myeloid cell inflammatory M1 polarization and inflammatory cytokines enhanced IL-27 expression in microglia and macrophages. Astrocytes in postmortem tissues and in vitro expressed IL-27R. Moreover, IL-27 triggered the phosphorylation of the transcription regulator STAT1, but not STAT3 in human astrocytes; indeed IL-27 up-regulated MHC class I expression on astrocytes in a STAT1-dependent manner. These findings demonstrate that IL-27 and its receptor are elevated in MS lesions and that local IL-27 can modulate immune properties of astrocytes and infiltrating immune cells. Thus, therapeutic strategies targeting IL-27 may influence not only peripheral but also local inflammatory responses within the brain of MS patients.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) characterized by demyelination, oligodendrocyte death, axonal loss, and glial cell activation (Sospedra and Martin 2005). The fundamental role of T lymphocytes in MS pathogenesis is well established (Denic et al. 2013; Sospedra and Martin 2005). A number of cytokines produced by T lymphocytes or acting on these immune cells have been implicated in the initiation and/or progression of MS and its animal models. Both pro-inflammatory cytokines, which enhance tissue destruction, and anti-inflammatory cytokines, which hinder inflammatory responses and/or promote repair, modulate MS pathobiology (Egwuagu and Larkin Iii 2013). Several lines of evidence suggest that interleukin-27 (IL-27) is a key cytokine modulating inflammatory and autoimmune diseases such as MS (Fitzgerald and Rostami 2009) .

IL-27 is a heterodimeric cytokine from the IL-12 family and is composed of the Epstein-Barr virus induced gene 3 (EBI-3) and p28 subunits. This cytokine is produced primarily by antigen-presenting cells in response to inflammatory mediators or microbial products (Pflanz et al. 2002). The signaling receptor of IL-27 (IL-27R) consists of two chains: glycoprotein 130 (gp130) and T-cell cytokine receptor (TCCR, also named WSX-1 or IL-27Rα) (Pflanz et al. 2004; Pflanz et al. 2002). The IL-27R is expressed on leukocytes including T lymphocytes, dendritic cells, and monocytes (Pflanz et al. 2004; Villarino et al. 2005). Although IL-27 was initially shown to promote the development of CD4 T helper (Th)1 responses (Pflanz et al. 2002), it is also demonstrated to limit Th1, Th2 and Th17 responses (Bosmann and Ward 2013; Wynick et al. 2014). IL-27 increases the proliferation and cytolytic functions of naive CD8 T lymphocytes by inducing transcription factor T-bet expression, IFNγ and granzyme B production (Mittal et al. 2012; Morishima et al. 2005; Schneider et al. 2011b).

Pleiotropic effects in chronic inflammatory and autoimmune diseases have been attributed to IL-27. While this cytokine can stimulate deleterious inflammatory responses in animal model of psoriasis (Shibata et al. 2013), it is also reported to impede the development of animal models of other diseases such as MS (Yoshida and Hunter 2015). IL-27 can inhibit the development of experimental autoimmune encephalomyelitis (EAE), the most commonly used animal model of MS (Batten et al. 2006; Fitzgerald et al. 2007a; Fitzgerald et al. 2007b; Goldberg et al. 2004; Stumhofer et al. 2006). Interestingly, the p28 subunit of IL-27 diminishes autoimmune responses in EAE and experimental autoimmune uveitis (Chong et al. 2014). The mechanisms involved include impaired Th17 cell development, increased differentiation of IL-10-producing regulatory T cells, and induction of CD39 on dendritic cells leading to their diminished capacity to promote Th1 and Th17 differentiation (Batten et al. 2006; Fitzgerald et al. 2007a; Fitzgerald et al. 2007b; Mascanfroni et al. 2013; Stumhofer et al. 2006). While IL-27 transcripts are detected in the CNS of EAE mice, the cell types producing this cytokine remain unknown (Li et al. 2005; Yan et al. 2008). Relevant to the human disease, the beneficial impact of IFNβ, a first-line therapy for relapsing-remitting MS, is associated with IL-27 induction, which promotes the production of IL-10 by dendritic cells (Sweeney et al. 2011; Zhang and Markovic-Plese 2010). Whether human neural cells could be an important source of IL-27 has not been investigated. Given this, identification of the sources, targets and functions of IL-27 in the context of different autoimmune diseases is deemed essential to refine the potential use of therapies either enhancing or blocking this cytokine.

In this report, we evaluate the expression of IL-27 in post-mortem human brain samples from MS patients and normal donors and report up-regulation of IL-27 in patients, especially on astrocytes and microglia/macrophages. We also establish that brain-infiltrating CD4 and CD8 T lymphocytes express IL-27R. Using *in vitro* human primary cell cultures, we confirm that glial

cells produce IL-27 in response to inflammatory stimulation. Moreover, we demonstrate that IL-1β+TNF treated astrocytes also up-regulate IL-27R expression and respond to IL-27 via STAT1 phosphorylation, resulting in increased MHC class I expression on these cells. Overall, our results demonstrate that IL-27 is produced locally in the brain of MS patients and that both resident (e.g. astrocytes) and infiltrating cells (e.g. T lymphocytes) can respond to this cytokine.

MATERIAL AND METHODS

Immunostaining of human brain samples

It was not possible to detect IL-27 and IL-27R on the same sections as IL-27 antibodies labeled cells only in formalin-fixed paraffin-embedded tissues whereas IL-27R antibodies were optimal for immunostaining in frozen tissues. Post-mortem brain tissue sections were obtained from tissue donors without clinical and neuropathological evidence of CNS disease (normal control samples) and patients diagnosed clinically and confirmed by neuropathological examination as having MS (Tables 1-2). Studies were approved by the CHUM Ethical Committee (BH07.001, HD07.002). Paraffin-embedded sections were deparaffinized, treated for antigen retrieval using sodium citrate buffer, and blocked for non-specific binding as previously published (Zaguia et al. 2013). Sections were also blocked for endogenous biotin using Avidin/biotin kit (Vector Laboratories, Burlington, ON, Canada) following the manufacturer's instructions. Primary antibodies recognizing IL-27 subunits (EBI-3, p28) and cell-specific marker for either astrocytes (glial fibrillary acidic protein, GFAP) or microglia/macrophages (ionized calcium-binding adapter molecule 1, Iba-1) were incubated overnight. All antibodies are listed in Table 3. Snap-frozen sections were obtained from the tissue bank NeuroResource, UCL Institute of Neurology, London, UK. Samples were donated to the tissue bank with written informed consent following ethical review by the London Research Ethics Committee, UK. Sections cut before and immediately after those used for immunofluorescence studies were also stained with oil red O (ORO) and hematoxylin for histopathological examination and scored as previously described (Pittet et al. 2011a). MS lesions were characterized using ORO and hematoxylin staining with scoring as active (AQ; containing numerous inflammatory cells and ORO-positive phagocytic macrophages that have engulfed myelin debris); subactive (SAQ; containing distinct demyelinated areas with fewer inflammatory cells and phagocytic macrophages); and chronic

plaques (CQ: demyelinated lesions without evidence of ongoing or recent demyelination). Snap-frozen sections were air-dried, fixed with either cold acetone or 4% (w/v) paraformaldehyde, blocked (Pittet et al. 2011a; Zaguia et al. 2013) and then incubated with antibodies specific for IL-27R chains (TCCR and gp130) and for CD4, CD8, or GFAP. Finally, both paraffin-embedded and snap-frozen sections were incubated with the nuclear staining TO-PRO®-3 iodide (Invitrogen™, Camarillo, CA, USA), treated with Sudan Black and mounted in Gelvatol as previously published (Pittet et al. 2011a; Pittet et al. 2011b; Zaguia et al. 2013). Control experiments were concurrently carried out on adjacent sections using isotype controls at the same concentration of corresponding primary antibodies. Slides were observed using a SP5 Leica confocal microscope and confocal images acquired simultaneously in different channels throughout multiple z-stacks using LASAF software.

Isolation and activation of human astrocytes

Human fetal CNS tissues from 14- to 20-week-old embryos were provided by the Albert Einstein College of Medicine (Bronx, NY, USA). The studies were approved by their and McGill University's ethical review boards. Purified astrocyte cultures were obtained as previously described (Saikali et al. 2007). Cells were treated for 24 (qPCR) or 48 h (FACS/ELISA/Western blot) with recombinant IFNγ (200 U/ml equivalent to 10ng/ml, Thermo Scientific, Rockford, IL, USA), TNF (2000 U/ml, Invitrogen), IL-1β (10 ng/ml, Invitrogen), or combinations of these cytokines. Astrocyte supernatants were collected after 48 hours and kept at -20°C until IL-27 was determined by ELISA according to the manufacturer's instructions (R&D Systems, distributed by Cedarlane Labs, Burlington, ON, Canada). For Western blot experiments addressing the impact of IL-27 on STAT1 and STAT3 phosphorylation, resting or IL-1β+TNF astrocytes (36-48h pretreatment with TNF+IL-1β) were exposed to 100 ng/ml of IL-27 for 15, 30 or 60 minutes. To assess the role of STAT1 signaling, astrocytes were either pretreated with Fludarabine (50μM) or

its diluent (DMSO) for 16h prior to the addition of IL-27 (100ng/ml) and then analyzed by flow cytometry 24 h later as described below.

Isolation and activation of human myeloid cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient (Schneider et al. 2011a; Schneider et al. 2011b). CD14 monocytes were then purified using CD14 beads (Miltenyi Biotec, San Diego, CA, USA) according to the manufacturer's instructions; purity assessed by FACS was >95%. Monocytes were matured into macrophages during a 7 day culture in Iscove medium supplemented with 10% (v/v) human serum, glutamine and antibiotics at a concentration of $1x10^6$ cells/ml as published by others (Brissette et al. 2012). Macrophages were subsequently stimulated with pro-inflammatory cytokines as described for astrocytes or with IFNγ (200U/ml) and LPS (100 ng/ml *E.coli* O127:B8, Sigma-Aldrich Saint Louis, MO, USA) for 48 h. These studies were approved by the ethical board of the CHUM (HD07.002, CE13.040) and a written informed consent was obtained from each healthy donor. Human fetal and adult microglia were isolated and polarized to the M1 or M2 phenotype as previously described (Moore et al. 2015). Supernatants were kept at -20^oC until IL-27 was determined by ELISA.

RNA isolation, reverse transcription, and qPCR

Total RNA was extracted using Qiazol (Invitrogen) and subsequently the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions and as previously described (Pittet et al. 2011a; Saikali et al. 2007). RNA samples were transcribed into cDNA using Quantitect Reverse Transcription kit according to the manufacturer's instruction (Qiagen). Relative gene expression levels were determined by quantitative real-time PCR (qPCR) using primers and TaqMan FAM–labeled probes for EBI-3 and p28 and VIC–labeled probe for

ribosomal 18S, the endogenous control, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Flow cytometry

Astrocytes were detached using warm PBS-EDTA (Pittet et al. 2011a; Saikali et al. 2007) and then labeled for surface HLA-ABC and intracellular GFAP molecules using antibodies listed on Table 2. To exclude dead cells, LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) was added simultaneously to the surface staining step. Appropriate isotype controls matched for concentration of primary antibodies were used in all steps. Samples were acquired on a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Treestar, Ashland, OR, USA).

Western blots

Cells were lysed and sonicated and extracted proteins were stored at −80°C; proteins were electrophoresed under reducing conditions and transferred onto PVDF membrane as previously published (Schneider et al. 2011b). Total and phosphorylated STAT1 and STAT3 proteins were quantified by chemiluminescence relative to actin using the Chemidoc MP imaging system (Bio-Rad Laboratories, Mississauga, ON, Canada). All antibodies are listed in Table 3.

Statistics

Data were analyzed using GraphPad Prism™ software (La Jolla, CA, USA). Results are represented as mean ± SEM. Statistical analyses included ANOVA followed by Bonferroni as post hoc test and paired Student's *t*-test. Values were considered statistically significant when probability (p) values were equal or below 0.05 (*), 0.01 (**), or 0.001 (***).

RESULTS

IL-27 and its receptor are highly expressed in MS brain lesions compared to control tissues

Multiple lines of evidence suggest that IL-27 is a key cytokine modulating autoimmune and inflammatory diseases such as MS (Hunter and Kastelein 2012; Rostami and Ciric 2013). However, whether this cytokine contributes to locally alter responses within the human brain remains unknown (Yoshida and Hunter 2015). Therefore, we sought to determine whether IL-27 and its receptor are preferentially present in the brain of MS patients. As IL-27 mRNA levels do not always predict protein expression (Antas et al. 2015), we performed immunohistochemistry to detect IL-27 protein subunits on brain tissues obtained from MS patients and controls (Table 1). Brain sections were stained for p28 (green) and EBI-3 (red) and adjacent sections were used for appropriate isotype controls. Six to 10 fields (at 430X, each field covering between 0.017 and 0.152 mm^2) per section were thoroughly analyzed to determine the number of cells per surface area expressing both subunits (representative fields are illustrated in Figure 1A). Although we detected cells positive for both subunits in all sections analyzed, the number and proportion as well as the signal intensity were markedly enhanced in MS lesions compared to control sections. Moreover, we observed that whilst most immunopositive cells expressed both subunits (Figure 1A), few of these cells expressed only the EBI3 subunit and none were only p28 immunopositive. Finally, our quantitative analysis confirmed that MS samples contain significantly more IL-27 expressing cells than normal control samples (Figure 1B; MS patients *vs.* controls: *** $p<0.001$). We also used Sudan black staining to identify demyelinated versus non-demyelinated areas and observed that most IL-27 expressing cells are localized in demyelinated or immediately adjacent areas. To validate that MS brain lesions express elevated levels of IL-27, we performed qRT-PCR for IL-27p28 on RNA extracted from post-mortem frozen samples from three MS lesions

and three controls. The relative IL27p28 mRNA levels were 8.8 higher in MS lesions compared to controls (Fig. 1C).

To determine whether cells present in the brain of MS patients might potentially respond to these elevated IL-27 levels, we investigated the expression of the IL-27R in post-mortem tissues obtained from MS patients and normal controls (see Table 2). Brain sections were stained for gp130 (red) and TCCR (green) and adjacent sections were used for appropriate isotype controls. Both chains were detected on the same cells (representative fields are illustrated in Figure 1D). Using Sudan Black staining we observed that double-positive cells are localized both in demyelinated areas and non-demyelinated areas. Finally, our quantitative analysis confirmed that MS sections contain significantly more IL-27R expressing cells per surface area than control sections (Figure 1E) (MS patients *vs.* controls: *$p<0.05$). Overall, our results demonstrate that both IL-27 and its receptor are elevated in MS brain lesions compared to controls.

IL-27 is strongly expressed by astrocytes and myeloid cells in MS lesions

We observed that IL-27 expressing cells exhibit various morphologies suggesting that several cell types could carry this cytokine. To identify these cells, we stained brain sections from controls and MS patients for p28 (in green) concomitantly for an astrocyte specific protein (GFAP in red) or a microglia/macrophage cell marker (Iba-1 in red) (Figure 2). Representative fields from two MS patients and one control shown for each staining (Figure 2A and C) illustrate that both GFAP or Iba-1 labeled cells can express p28. Whereas only a fraction of astrocytes exhibited detectable p28 in control sections, most astrocytes expressed this cytokine subunit in MS lesions. The proportion of astrocytes expressing p28 (Figure 2B: %GFAP+p28+ cells amongst GFAP) as well as the number of p28-expressing astrocytes per surface area (Figure 2B: GFAP+p28+/mm^2) are statistically more elevated in MS lesions compared to controls (Figure 2B; MS patients *vs.* controls: ***$p<0.001$). As expected (Bruck et al. 1996), we observed a

considerable increased in the number of Iba-1 positive cells per surface areas in MS lesions compared to controls (Figure 2E). The percentage of p28 expressing microglia/macrophage is similar in sections from controls and MS patients; however, as these myeloid cells are much more abundant in MS lesions, they locally provide more IL-27 per surface area (Iba+p28+/mm^2) than in controls (Figure 2E). To assess whether astrocytes and myeloid cells account for most IL-27 immunodetection in MS brain tissues, we combined the detection of GFAP+Iba-1 in one channel and assess p28 localization (Figure 2D). Although we could detect cells that were either astrocytes or microglia/macrophages but did not express p28, we could not find p28-expressing cells that were negative for both cell markers (Iba-1 and GFAP). Our results support the notion that most of the detectable IL-27 in MS tissues is provided by myeloid and astocytic cells. Overall, these data establish that the number of glial cells (astrocytes and microglia) and infiltrating macrophages producing IL-27 is significantly elevated in MS patients compared to controls.

Pro-inflammatory conditions increase IL-27 expression by human astrocytes and myeloid cells

We evaluated whether inflammatory conditions that are prevalent in MS lesions can trigger the secretion of IL-27 by human astrocytes. Primary cultures of human astrocytes were either untreated (Nil) or activated with different pro-inflammatory cytokines: IFNγ, IL-1β, TNF, or combination of those found in the brain of MS patients (Figure 3), and IL-27 expression at the mRNA and protein levels was assessed. The mRNA levels of both EBI-3 and p28 were low in untreated astrocytes (Figure 3A) but treatments with cytokine combinations, especially IFNγ+TNF, significantly increased these levels. We observed that supernatants collected from astrocytes under basal conditions contained very low amounts of IL-27 (161 pg/ml on average); however, considerable amounts (average from 638 to 1084 pg/ml) of this cytokine were detected

following treatments with TNF, IFNγ+TNF or TNF+IL-1β (Figure 3B). These results demonstrate that inflammatory cytokines trigger the expression of IL-27 by human astrocytes.

We also assessed whether myeloid cells: monocyte-derived human macrophages and microglia secrete IL-27 in response to pro-inflammatory conditions. While untreated human macrophages secreted low levels of this cytokine (268 pg/ml on average) (Figure 3C) these cells produced significantly elevated levels of IL-27 in response to the inflammatory cytokines IL-1β or TNF (Figure 3C) reaching levels (2,000-2,465 pg/ml) slightly lower than those induced by the strong IFNγ+LPS positive control (3,500 pg/ml) (Figure 3C). Previous studies have shown that murine astrocytes and microglia increase their IL-27 production in response to IFNγ + LPS stimulation (Fitzgerald et al. 2007a; Sonobe et al. 2005). We also evaluated whether human microglia and macrophages exhibiting different phenotypes: unpolarized or M1/M2 polarized cells could produce IL-27. We found that pro-inflammatory M1 polarized myeloid cells produce significantly greater levels of IL-27 compared to unpolarized or anti-inflammatory M2 counterparts (M1 fetal microglia compared to unpolarized or M2 polarized cells *$p<0.05$, M1 adult microglia or adult macrophages compared to their unpolarized or M2 polarized counterparts ***$p<0.001$) (Figure 3D). These data show that both human microglia and macrophages, which are abundantly present in MS lesions, produce IL-27 in response to inflammatory conditions.

Brain infiltrating CD8 and CD4 T lymphocytes express IL-27R

We and others have previously demonstrated that a proportion of peripheral blood human CD4 and CD8 T lymphocytes expresses the IL-27R chains: gp130 and TCCR (Charlot-Rabiega et al. 2011; Schneider et al. 2011b). We investigated whether brain-infiltrating T lymphocytes found in MS lesions express the IL-27R specific chain TCCR and could then potentially respond to the elevated IL-27 levels we observed (Figures 1-2). We could detect in post-mortem human brain tissues co-localization of either CD4 or CD8 (in red) and TCCR (in green) (Figure 4). As

expected, only rare T lymphocytes were detected in control brain tissues, but both CD4 and CD8 T cell subsets were more abundant per surface area in MS lesions (Figure 4B and D) (Wu and Alvarez 2011). The percentage of CD4 or CD8 T lymphocytes expressing TCCR varied from 25 to 100% and was similar in MS and control groups. This proportion of TCCR-expressing T cells is elevated compared to the average 1-7% of circulating human T lymphocytes from healthy donors carrying this receptor (Schneider et al. 2011b). These observations suggest that the proportion of brain infiltrating T cells equipped to respond to IL-27 is enhanced compared to the peripheral blood counterparts. The number of TCCR-expressing CD4 or CD8 T lymphocytes per surface area was significantly increased in MS lesions compared to controls (Figure 4B, D). Moreover, we observed TCCR-expressing T cells in perivascular cuffs but also in lesion parenchyma, suggesting that these brain-infiltrating T lymphocytes could be exposed to IL-27 provided by astrocytes or myeloid cells. Overall, our results suggest that an elevated proportion of brain-infiltrating T lymphocytes express IL-27R.

Myeloid cells in MS lesions express IL-27R

We and others showed that human monocytes and macrophages can express IL-27R (Robinson and Nau 2008; Schneider et al. 2011b). Therefore, we assessed whether myeloid cells present in MS lesions could also express the IL-27R. Indeed, we observed co-localization of TCCR (green), the IL-27R specific chain, with the Iba-1 (red) labeling in post-mortem human brain tissues obtained from three MS patients; one example is illustrated in Figure 4E. Although many Iba-1 immunoreactive cells expressed TCCR, it was possible to detect myeloid cells not carrying detectable levels of this receptor chain.

Astrocytes express IL-27R and respond to IL-27

Amongst the cells expressing both IL27R chains in post-mortem human brain tissues, we observed cells with a lymphoid shape but also other cells exhibiting an astrocyte-like shape. Thus, we evaluated whether astrocytes could also express the IL-27R. We detected co-localization of the IL-27R specific chain TCCR (green) with the GFAP (red) labeling in post-mortem human brain tissues obtained from both MS patients and controls (Figure 5A). Moreover, primary cultures of astrocytes treated with pro-inflammatory cytokines (e.g. TNF+IL-1β or IFNγ+TNF) expressed enhanced levels of TCCR and gp130 as detected by immunocytochemistry (Figure 5B). Collectively, these results demonstrate that human astrocytes can express both chains of the IL-27R especially in inflammatory conditions.

It is well documented that following binding to its receptor, IL-27 triggers the phosphorylation of STAT1 and STAT3 in immune cells (Hunter and Kastelein 2012; Iwasaki et al. 2015) as well as in non-immune cells such as keratinocytes (Wittmann et al. 2009). We have previously shown that IL-27 (100 ng/ml) quickly induces the phosphorylation of STAT1 and STAT3 in naive CD8 T lymphocytes, peaking at 15 minutes post-exposure to the cytokine (Schneider et al. 2011b). Therefore, we investigated whether astrocytes respond to IL-27 in a similar fashion. Moreover, as IL-1β+TNF treated astrocytes expressed elevated amount of IL-27R compared to the untreated cells, we assessed whether IL-27 triggered the phosphorylation of STAT1 and STAT3 in resting and IL-1β+TNF treated by western blot. We detected both STAT1 and STAT3 proteins in astrocyte lysates (Figure 5C). One typical example is illustrated whereas quantification of three samples is shown (Figure 5D). We found that the IL-1β+TNF pre-treatment markedly increased STAT1 protein levels, whereas it did not affect STAT3 expression (Figure 5C). Addition of IL-27 rapidly induced phosphorylation of STAT1, peaking at 15 and 30 minutes. However, although astrocytes expressed STAT3, we did not observe an induction of STAT3 phosphorylation in astrocytes following IL-27 exposure. Therefore, in contrast to other

immune and non-immune cells, IL-27 did not trigger STAT3 signaling in astrocytes. However, resting astrocytes responded to IL-27 by phosphorylating STAT1; IL-1β+TNF treated astrocytes expressed greater levels of STAT1 and consequently responded with an enhanced STAT1 phosphorylation compared to resting cells. These observations suggest that IL-27 triggers in human astrocytes a distinct pattern of signaling pathways compared to other cell types.

IL-27 increases MHC class I expression by astrocytes in STAT1 dependent manner

As IL-27 triggered cell signaling in human astrocytes, we investigated whether this cytokine could also shape immune properties of these cells. In different cell types IL-27 can induce or increase the expression of several molecules including MHC class I (Charlot-Rabiega et al. 2011; Feng et al. 2008) in a STAT1 dependent manner (Kamiya et al. 2004). Therefore, we assessed whether IL-27 triggers the expression of MHC class I via STAT1 signaling in astrocytes. Primary cultures of human astrocytes were either pretreated with fludarabine, a STAT-1 specific inhibitor that does not affect other STATs (Frank et al. 1999; Furukawa et al. 2009) or the control diluent (DMSO) prior to be exposed to IL-27. We observed that the percentage of astrocytes expressing detectable levels of MHC class I molecules significantly increased following IL-27 treatment (Figure 5E-F) and that this enhanced expression was abrogated when astrocytes were pretreated with fludarabine. Therefore, our results demonstrate that IL-27 can shape astrocyte immune functions such as MHC class I expression in a STAT1 dependent manner.

DISCUSSION

While the capacity of IL-27 to inhibit the development and progression of the most common animal model of MS is well established (Batten et al. 2006; Fitzgerald et al. 2007a; Fitzgerald et al. 2007b; Goldberg et al. 2004; Stumhofer et al. 2006), whether this cytokine can exert its impact directly in the CNS is still under investigation. Moreover, an increasing amount of data supports the notion that IL-27 can dampen immune responses in the peripheral blood of MS patients (Severa et al. 2015; Sweeney et al. 2011; Tang et al. 2015) but whether this cytokine locally modulates immune responses within the human CNS has not been previously investigated. In the present study, we demonstrate that the expression of IL-27 is elevated in MS lesions compared to normal control brain tissues and that astrocytes and microglia/macrophages are abundant sources of this cytokine. Moreover, an important proportion of brain infiltrating T lymphocytes bear the IL-27R supporting the notion that these lymphocytes could potentially respond to IL-27. Finally, we document that human astrocytes can also express IL-27R and respond to this cytokine.

We observed an excellent co-localization of both IL-27 subunits EBI-3 and p28 in post-mortem brain samples (Figure 1) especially in MS lesions. Very few cells (less than 10%) were positive for EBI3 only and we could not detect p28-expressing cells that were EBI3 negative. Our results suggest that the p28 and EBI3 proteins combine in the brain of MS patients to form IL-27, but we cannot rule out that these subunits could also associate with other partners, or may be present as individual proteins. EBI3 can bind to the IL-12p35 subunit to form IL-35 (Collison et al. 2007; Devergne et al. 1997). Human microglia and macrophages can produce both IL-12 subunits (Jack et al. 2005; Li et al. 2007). Moreover, p28 can form a complex with cytokine-like factor 1 (CLF-1) (Crabe et al. 2009); human macrophages under inflammatory conditions have been shown to express CLF-1 (Kass et al. 2012). Although human astrocytes can produce the IL-

12p40 subunit (Burns et al. 2012; Constantinescu et al. 1996), whether they can also express p35 is not resolved and no data is available pertaining to CLF-1 production by astrocytes. Therefore, it remains possible that p28 or EBI3, especially in myeloid cells, combines with other partners.

We detected greater numbers of IL-27 expressing astrocytes and microglia/macrophages per surface area in MS lesions compared to control tissues (Figure 2). Similarly, elevated levels of IL-27 have been detected in the CNS of mice during EAE (Fitzgerald et al. 2007a; Haroon et al. 2011; Li et al. 2005; Yan et al. 2008; Yang et al. 2012) or following infections such as *Toxoplasma gondii* and mouse hepatitis virus strain JHM (de Aquino et al. 2014; Stumhofer et al. 2006). Therefore, we suggest that the presence of enhanced IL-27 levels within the inflamed CNS is a common feature of several pathological conditions. As primary cultures of rodent microglia (Holley et al. 2012; Lee et al. 2011; Sonobe et al. 2005; Xu and Drew 2007; Xu et al. 2007) and astrocytes (Fitzgerald et al. 2007a; Gorina et al. 2011; Xu and Drew 2007) produce IL-27 in response to inflammatory mediators, we also tested whether the human counterparts could also secrete this cytokine in vitro. We demonstrated that TNF and IL-1β, which are upregulated in the CNS of patients with MS or other neurological diseases (Montgomery and Bowers 2012; Rothwell and Luheshi 2000; Simi et al. 2007), trigger IL-27 secretion by astrocytes and macrophages (Figure 3). Our results confirm a previous report establishing that TNF induces IL-27 production by human macrophages (Kalliolias et al. 2010); we also detected a similar TNF impact on human astrocytes (Figure 3). Other studies have demonstrated that IFNγ can trigger IL-27 production by murine macrophages and astrocytes (Hindinger et al. 2012; Liu et al. 2007) but we could not detect this effect on human macrophages and astrocytes (Figure 3B and D). Moreover, IFNγ did not significantly increase TNF- or IL-1β-induced IL-27 secretion by both cell types (Figure 3B and C). This discrepancy could be due to specific species attributes (mouse vs. human) or to a dose effect as Hindinger and colleagues (Hindinger et al. 2012) used a 10-fold

higher dose (100 ng/ml) of IFNγ than we did (10 ng/ml). We also showed that M1 polarizing conditions significantly increase the secretion of IL-27 by human macrophages and microglia (Figure 3D). Interestingly, myeloid cells bearing M1 and M2 associated markers have been detected in MS lesions (Miron et al. 2013; Peferoen et al. 2015). Overall, our results suggest that the human astrocytes and myeloid cells can locally produce IL-27 in response to specific inflammatory stimuli.

Increased TCCR mRNA levels have been reported in the CNS of mice during EAE or CNS infections although the cell types were not identified (de Aquino et al. 2014; Li et al. 2005). Monocytes and macrophages are amongst the numerous human cell types that can express the IL-27R (Pflanz et al. 2004; Robinson and Nau 2008; Schneider et al. 2011b). Indeed, we observed that microglia/macrophages in MS brain tissues express TCCR (Figure 4). IL-27 can favor both proinflammatory and anti-inflammatory responses by human myeloid cells (Guzzo et al. 2012a; Guzzo et al. 2010; Kalliolias et al. 2010; Robinson and Nau 2008). We have established that brain infiltrating CD4 and CD8 T lymphocytes in human post-mortem tissues express the IL-27R (Figure 4) supporting the notion that these cells could potentially respond to the enhanced IL-27 levels observed in MS brain. Numerous publications have documented the impact of IL-27 on multiple T cell functions and properties (Yoshida and Hunter 2015). IL-27 can dampen the capacity of human T lymphocytes to produce cytokines that have been implicated in the pathogenesis of MS (Codarri et al. 2013; Kebir et al. 2009; Kebir et al. 2007): IL-17, IL-22 and GM-CSF (Liu and Rohowsky-Kochan 2011; Young et al. 2012). IL-27 can also boost the expression of PD-L1 by T lymphocytes (Hirahara et al. 2012) and favor the development of regulatory T cells (Do et al. 2015; Yoshida and Hunter 2015). Moreover, IL-27 can up-regulate the production of IL-10 by several mouse and human CD4 and CD8 T cell subsets (Ansari et al. 2011; Awasthi et al. 2007; Fitzgerald et al. 2007b; Murugaiyan et al. 2009; Perona-Wright et al.

2012; Stumhofer et al. 2007; Wang et al. 2011; Wang et al. 2013; Yoshida and Hunter 2015). Such IL-27 effects on IL-10, a key cytokine reducing the production of several proinflammatory cytokines (e.g. TNF, GM-CSF, etc.) but favoring the production of anti-inflammatory molecules (reviewed in (Ireland et al. 2015)), could potentially have an impact on the ongoing CNS inflammatory responses in MS patients. Notably, we and others have shown that the IL-27-mediated effects on T lymphocytes are shaped by the activation or differentiation status of the targeted cells (Schneider et al. 2011b; Yoshida and Hunter 2015). For example, while IL-27 can induce a robust production of IL-10 by naïve or activated CD8 T cells, such impact is impaired in memory CD8 T cells from mice and humans (Perona-Wright et al. 2012). Additional investigations will be necessary to characterize the impact of local CNS sources of IL-27 on the infiltrating T lymphocytes in the context of MS.

Our results demonstrate that human astrocytes both in tissue and in vitro can express the IL-27R chains especially in inflammatory conditions (Figure 5). Other human cell types including monocytes and macrophages have been shown to be able to both produce and respond to IL-27 (Dai et al. 2013; Guzzo et al. 2012a; Guzzo et al. 2010; Guzzo et al. 2012b). IL-27 triggers the phosphorylation of STAT1 and STAT3 in several cell types including human myeloid cells and T lymphocytes (Pflanz et al. 2004; Wynick et al. 2014). Although, we could easily detect STAT3 and STAT3p in astrocyte lysates, we could not observe any IL-27-mediated STAT3 phosphorylation at the time points we tested (15, 30 and 60 min; Figure 5C-D). In contrast, IL-1β+TNF treated astrocytes express not only elevated IL-27R levels (Figure 5B) but also significantly enhanced STAT1 protein levels compared to resting counterparts (Figure 5C-D). Moreover, IL-27 triggers a rapid phosphorylation of STAT1 in both resting and IL-1β+TNF treated astrocytes; not surprisingly STAT1p levels are greater in IL-1β+TNF treated cells (Figure 5C-D). A recent study compared the contribution of STAT1 vs. STAT3 signaling to the

transcriptional pattern induced by IL-27 and IL-6 in CD4 T cells and the authors concluded that STAT1 could not compensate for STAT3-driven effects but that STAT1 signaling was responsible for a subset of genes triggered by IL-27 and not IL-6 (Hirahara et al. 2015). Our results suggest that in contrast to T cells, IL-27 triggered signaling pathways in human astrocytes do not include STAT3. In contrast, IFN-γ has been shown to trigger the phosphorylation of both STAT1 and STAT3 in human astrocytes (Hashioka et al. 2011). Moreover, LPS and oncostatin M could trigger STAT3 phosphorylation in rodent astrocytes (Baker et al. 2008; Morga et al. 2009). Similarly, following an optic nerve crush the activation of Müller cells, which are retinal glial cells, has been shown to depend on a gp130-signaling cytokine (leukemia inhibitory factor) which induces STAT3 phosphorylation (Kirsch et al. 2010). Others have demonstrated that IL-27 enhances MHC class I expression in human endothelial cells (Feng et al. 2007); similarly we observed that IL-27 enhanced the expression of these molecules in human astrocytes and that fludarabine pretreatment prevented this upregulation suggesting a STAT1-mediated effect (Figure 5). Finally, we observed that IL-27 did not induce the production of IL-6 but triggered the secretion of CXCL10 by mechanisms that are not inhibited by fludarabine (data not shown) supporting the notion that IL-27 induces other signaling pathways than STAT1. We are currently investigating additional signaling pathways triggered by IL-27 in human astrocytes. Overall, our results demonstrate that IL-27 is not only produced by human astrocytes but also modulate the properties and functions of these cells.

In animal models, gp130, the broadly used receptor chain, has been shown to play a role in astrocyte functions. Mice lacking gp130 specifically on astrocytes developed EAE with enhanced severity, augmented inflammatory cytokine levels and decreased number of regulatory T cells within the CNS compared to wild type animals (Haroon et al. 2011). Similarly, these astrocyte-gp130 deficient mice exhibited diminished survival following induction of Toxoplasma

encephalitis (Drogemuller et al. 2008). Unfortunately, no investigation has addressed the role of TCCR specifically on astrocytes using in vivo models. However, the unique contribution of IL-27 to local immune responses in an immune-privileged organ has been illustrated in a model of uveitis. IL-27 and its receptor are constitutively expressed and are upregulated in inflammatory conditions (e.g. IFNγ, uveitis) by microglia and retinal cells respectively (Amadi-Obi et al. 2007; Lee et al. 2011). Moreover, this local IL-27 has been shown to dampen the expansion of Th17 cells and induce IL-10 production in photoreceptors (Amadi-Obi et al. 2007; Lee et al. 2011). Therefore, we can speculate that the brain IL-27 and IL-27R expression we observed in MS patients modulate local inflammatory responses. As astrocytes can both limit and promote inflammatory and disease processes (Johann et al. 2015; Molofsky and Deneen 2015; Sofroniew 2015) and that IL-27 triggers a unique signaling cascade in these cells, further investigations will be necessary to elucidate the impact of IL-27 provided not only by astrocytes but also myeloid cells in the context of MS.

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Footnotes

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Table 1: Description of paraffin embedded post-mortem brain tissues

Block	M/F	Age (Y)	DD (Y)	Sample type	Infiltrate (1-5)	Summary observations from autopsy report
CTL-1	M	51	n.a.	NC, Brain	n.a.	Normal white and grey matter
CTL-2	F	46	n.a.	NC, Brain	n.a.	Normal white and grey matter
CTL-3	M	58	n.a.	NC, Brain	n.a.	Normal white and grey matter
CTL-4	M	34	n.a.	NC, Brain	n.a.	Normal white and grey matter
CTL-5	M	72	n.a.	NC, Brain	n.a.	Normal white and grey matter
MS-1	M	42	21	AQ, Brain	3	Numerous demyelinated plaques
MS-2	M	42	21	AQ, Brain	4	Numerous demyelinated plaques around vessels
MS-3	M	47	27	AQ, Brain	2-3	Several demyelinated plaques
MS-4	M	42	15	AQ, RCO	3	One very large plaque and additional area of demyelination
MS-5	F	66	19	AQ, Brain	2	Several demyelinated plaques
MS-6	F	66	19	AQ, Brain	3	Several demyelinated plaques, infiltrates in and outside plaques
MS-7	F	68	0.6	AQ, Brain	1	One demyelinated plaque
MS-8	F	52	8	SAQ, Cx, DGM	n.d.	Multiple demyelinated plaques and areas of remyelination
MS-9	F	60	25	CQ, pons	n.d.	Few demyelinating lesions

MS-10	M	55	0.08	AQ, M, GM	n.d.	One demyelinating lesion
MS-11	M	55	0.08	AQ, M, GM	n.d.	Few demyelinating lesions
MS-12	F	45	12	SAQ, Cx, WM	n.d.	Few demyelinating lesions and one area of remyelination

Abbreviations: CTL: control subject, MS: MS patient, DD: disease duration, n.a. non applicable; NC: Normal control, AQ: active plaque, SAQ: subactive plaque; CQ: Chronic plaque, RCO: right centrum ovale, DGM: deep grey matter, GM: grey matter, WM: white matter, Cx: cortex; M: medulla

Table 2: Description of sections for immunostaining cut from snap-frozen post-mortem brain samples

Block	M/F	Age (Y)	DD (Y)	Sample type	ORO; Hemaoxylin (0-5) score	Summary observations from Hemaoxylin-ORO stained sections
CTL-A	M	49	n.a.	NC W & G, PV, R	0;0	Normal control white and grey matter
CTL-B	F	75	n.a.	NC W, FV, L	0;0	Normal control white matter
CTL-C	M	28	n.a.	NC W, PSV, L	0;0	Normal control white matter
CTL-D	M	53	n.a.	NC W, OSv, R	0;0	Normal control white matter
MS-A	F	47	20	AQ, FSv, L	4;3	White and grey matter surrounding active plaque, ORO_+ cells in blood vessel walls and parenchyma
MS-B	F	37	10	SAQ, OV, R	2;2	Subactive demyelinating plaque
MS-C	F	60	34	AQ, TV, L	3;4	Four demyelinating plaques with many large and small perivascular cuffs
MS-D	F	29	8	SAQ, Pons, R	2;2	Two subactive demyelinating plaques
MS-E	M	35	0.8	NAWM, OV, L	0;2	MS white matter with perivenular cuffing without demyelination
MS-F	F	55	22	AQ, CGM, L	4;4	Four demyelinating plaques

MS-G	F	43	22	AQ, P, L	3;3	Two active demyelinating plaques
MS-H	F	47	20	AQ, PSv, L	5;4	Large plaque with active and some subactive and chronic areas. Large perivascular cuffs.
MS-I	F	37	10	AQ, basal ganglia, L	3;3	Large subactive plaque with perivascular cuffing, areas of grey matter
MS-J	F	49	11	SAQ, F pole V, R	0;2	Large chronic plaque surrounded by pale abnormal white matter

Abbreviations:

CTL: control subject, MS: MS patient, DD: disease duration, NC: normal control, AQ: active plaque, SAQ: subactive plaque, W: white matter, G: grey matter, NAWM: normal appearing white matter, V: ventricular, Sv: subventricular, F: frontal, P: parietal, T: temporal, O: occipital, R: right, L: left. ORO and cuffing and haematoxylin staining scored on a scale of 0 to 5; 0 is what would be expected in normal control brain tissues.

Table 3: List of reagents used to detect human proteins

Reagent	Application[1]	Source
Rabbit α-EBI3	IHF	Abcam
Goat α-IL-27p28	IHF	R&D Systems
Rabbit α-GFAP	IHF	Dako
Rabbit α-Iba1	IHF	Wako
Goat α-gp130	IHF	R&D Systems
Rabbit α-TCCR	IHF	LifeSpan BioSciences
Goat α-TCCR	IHF	R&D Systems
Rabbit α-CD8 (clone SP16)	IHF	Vector Laboratories
Mouse α-CD4 (clone CA4)	IHF	Abcam
Rhodamine Red™-X-conjugated donkey α-rabbit	IHF	Jackson ImmunoResearch
Biotinylated rabbit α-goat	IHF	Dako
Alexa Fluor® 488-conjugated streptavidin	IHF	Life Technologies
Biotinylated donkey α-goat	IHF	Jackson ImmunoResearch
Cy™3-conjugated streptavidin	IHF	Jackson ImmunoResearch
Alexa Fluor® 488-conjugated goat α-rabbit	IHF	Life Technologies
Cy™3-conjugated donkey α-mouse	IHF	Jackson ImmunoResearch
Pacific Blue-conjugated mouse α-HLA A,B,C	FACS	BioLegend
Alexa Fluor® 488-conjugated mouse α-GFAP	FACS	Invitrogen
Rabbit α-STAT1	Western blot	Cell Signaling Technology
Mouse α-phospho STAT1 (Tyr701) (clone M135)	Western blot	Abcam
Mouse α-STAT3 (clone 124H6)	Western blot	Cell signaling
Rabbit α-phospho STAT3 (Tyr705) (clone D3A7)	Western blot	Cell signaling Technology

Mouse α-actin (clone C4)	Western blot	MP Biomedical
HRP-conjugated donkey α-rabbit	Western blot	Jackson ImmunoResearch
HRP-conjugated rabbit α-mouse	Western blot	Dako
Biotinylated goat α-rabbit	Western blot	Dako

[1]IHF: Immunohistofluorescence; FACS: flow cytometry

Figure legends

Figure 1: The expression of IL-27 and its receptor is elevated in MS brain tissues compared to controls.

Expression of IL-27 (A, B, C) and IL-27R (D, E) in brain samples obtained from MS patients and controls. (A) Representative photomicrographs of co-staining for IL-27 p28 (green) and EBI-3 (red) subunits or (D) IL-27R TCCR (green) and gp130 (red) subunits in two MS and one control samples. Nuclei were stained with TO-PRO®-3 iodide (blue). Corresponding isotype controls are shown (Iso). Scale bar values=25µm. White boxes delineate the enlarged areas which are illustrated in the last column. (B, E) Quantification of the number of (B) EBI-3$^+$p28$^+$ cells per surface (mm^2) or (E) gp130$^+$TCCR$^+$ cells per surface (mm^2) in human brain sections from MS lesions and controls. Each dot represents one donor, and for each donor 6 to 10 fields were analyzed. Student's *t*-test comparing MS lesion tissues vs. controls; *** $p< 0.001$, * $p< 0.05$. C) mRNA relative expression of p28 IL-27 subunit in post-mortem brain tissues from controls or MS lesions. Data pooled from 3 donors.

Figure 2. IL-27 is produced by astrocytes and microglia/macrophages in MS brain tissues.

Expression of IL-27 p28 subunit by astrocytes(A) and microglia/macrophages (C) in brain samples from MS patients and controls. Representative photomicrographs of co-staining for IL-27 p28 (green) and either GFAP (red, demonstrating astrocytes) (A) or Iba-1(showing macrophages and microglia) (C) in two MS samples and one control sample. Nuclei were stained with TO-PRO®-3 iodide (blue). Corresponding isotype controls are shown (Iso). Scale bar values=25µm. White boxes delineate the enlarged areas which are illustrated in the last column. (B) Percentage of p28+ expressing astrocytes as well as quantification of the number of p28+ expressing astrocytes per surface area in sections from MS lesions and controls. (D) Astrocytes and microglia/macrophages represent most IL-27 sources. Detection of IL-27 p28 (green) and glial cells (GFAP and Iba-1, red) in one MS lesion representative of two tested. (E) Quantification of p28$^+$ expressing microglia/macrophages and quantification of microglia/macrophages per surface area in sections from MS lesions and control tissues. (B, E) Each

dot represents one donor, and for each donor 6 to 10 relevant pictures were analyzed. Student's *t*-test comparing MS tissues vs. normal controls *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 3. Pro-inflammatory stimuli up-regulate IL-27 expression by human astrocytes, microglia and macrophages.

(A, B) Human astrocytes were either untreated (Nil) or stimulated with the indicated cytokines (IFNγ, TNF, IL-1β or in combinations) for either 24h (A) or 48 h (B) and IL-27 expression was determined by qPCR (A) or ELISA (B). (A) mRNA relative expression of EBI3 and p28 IL-27 subunits by astrocytes. Data pooled from 6 donors. (B) ELISA analysis of IL-27 secretion by astrocytes. Data pooled from 6 donors. (C, D) IL-27 production assessed by ELISA in supernatants from activated human microglia or macrophages. (C) Human macrophages were either untreated (Nil) or stimulated with different cytokines for 48h. Data are pooled for n=7 donors. (D) Human fetal or adult microglia and adult macrophages were either unpolarized (M0), or polarized into M1 or M2 phenotype and supernatants collected after 48h stimulation. Data are pooled for n=5 donors. One way ANOVA and Bonferroni's multiple comparison test. Untreated vs. treated cells (A, B, C) or M1 cells vs. M0 or M2 cells (D) *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 4. Brain–infiltrating T lymphocytes and myeloid cells express IL-27R (TCCR subunit)

Expression of the IL-27R TCCR chain by T lymphocytes and microglia/macrophages in brain samples obtained from MS patients and controls. (A) Representative photomicrographs of co-staining for TCCR IL-27R chain (green), and either (A) CD4 (red) or (C) CD8 (red), and nuclei (blue) in two MS and one control samples. Corresponding isotype controls are shown (Iso). Scale bar values=25μm. White boxes delineate the enlarged areas which are illustrated in the last column. (B, D) Number of TCCR positive CD4 (B) and CD8 (D) T lymphocytes per surface (mm^2) and number of CD4 (B) and CD8 (D) T lymphocytes per surface area in human brain sections from MS lesions and controls. Each dot represents one tissue block, and for each tissue 6 to 10 relevant pictures were analyzed. Student's *t*-test comparing MS lesion tissue vs. controls sections *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (E)

Representative photomicrographs of co-staining for TCCR IL-27R chain (green), and Iba-1 (red) and nuclei (blue) in one MS patient. Similar results were obtained for two additional MS donors.

Figure 5. Astrocytes express the IL-27R and respond to the cognate cytokine

(A) Expression of the IL-27R TCCR chain by astrocytes in brain samples from MS patients and controls. Representative micrographs of co-staining for TCCR IL-27R chain (green), GFAP (red) and nuclei (blue). Corresponding isotype controls are shown. Scale bar values=25µm. White boxes delineate the enlarged areas which are illustrated in the last column. (B) Expression of IL-27R chains by primary cultures of human astrocytes. Astrocytes were either untreated (Nil) or stimulated with TNF+IL-1β for 48 hours and then analyzed by immunocytochemistry for TCCR (green) and gp130 (red). (C, D) IL-27 triggers STAT1 phosphorylation but not STAT3 phosphorylation in human astrocytes. Astrocytes were either pretreated with pro-inflammatory cytokines TNF+IL-1β (cytokine treated) or not (resting) for 48 hours prior to being exposed to IL-27 (100 ng/ml) for specific time points (0, 15, 30 and 60 min). Western blot analysis (n=3) of total and phosphorylated STAT1 and STAT3 expression. One representative Western blot (C) and quantification as relative expression compared to actin for 3 independent donors (D) are shown. STAT1 total resting vs. IL-1β+TNF-treated astrocytes *** $p < 0.001$. STAT1p IL-1β+TNF-treated astrocytes 0min vs. 15 min ** $p < 0.01$ and 0 min vs. 30 min *** $p < 0.001$. (E, F) Inhibition of STAT1 prevents IL-27-mediated MHC class I upregulation. Astrocytes were either pretreated with fludarabine or its control diluent (DMSO) prior to the culture in the presence or absence of IL-27 (100 ng/ml) for 24 hours. Subsequently, cells were collected, stained for HLA-ABC and LIVE/DEAD Cell stain or isotype controls and analyzed by flow cytometry. Only living single cell events were analyzed. Representative dotplots are illustrated (E) as well as (F) quantification of experiments performed on cells from 6 donors. DMSO+IL-27 treatment vs. all other treatments were *** $p < 0.001$.