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Activation of mixed glia by Aβ-specific Th1 and Th17 cells and its regulation by Th2 cells

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ABSTRACT

Microglia are innate immune cells of the CNS, that act as antigen-presenting cells (APC) for antigen-specific T cells and respond to inflammatory stimuli, such as amyloid-beta (Aβ), resulting in the release of neurotoxic factors and pro-inflammatory cytokines. Astrocytes can also act as APC and modulate the function of microglia. However, the role of distinct T cell subtypes, in particular Th17 cells, in glial activation and subsequent modulatory effects of Th2 cells are poorly understood. Here, we generated Aβ-specific Th1, Th2, and Th17 cells and examined their role in modulating Aβ-induced activation of microglia in a mixed glial culture, a preparation which mimics the complex APC types in the brain. We demonstrated that mixed glia acted as an effective APC for Aβ-specific Th1 and Th17 cells. Addition of Aβ-specific Th2 cells suppressed the Aβ-induced IFN-γ production by Th1 cells and IL-17 production by Th17 cells with glia as the APC. Co-culture of Aβ-specific Th1 or Th17 cells with glia markedly enhanced Aβ-induced pro-inflammatory cytokine production and expression of MHC class II and co-stimulatory molecules on the microglia. Addition of Aβ-specific Th2 cells inhibited Th17 cell-induced IL-1β and IL-6 production by mixed glia and attenuated Th1 cell-induced CD86 and CD40 expression on microglia. The modest enhancement of MHC class II and CD86 expression on astrocytes by Aβ-specific Th1 and Th17 was not attenuated by Th2 cells. These data indicate that Aβ-specific Th1 and Th17 cells induce inflammatory activation of glia, and that this is in part regulated by Th2 cells.

1. Introduction

Microglia are members of the macrophage family and key mediators of innate immune responses in the CNS. Evidence is emerging that microglial activation is a fundamental feature of neurodegenerative diseases, such as Alzheimer's disease (AD). The neuritic plaques found in AD patients are associated with activated microglia, and in particular with enhanced expression of MHC class II (Luber-Narod and Rogers, 1988; Rogers et al., 1988). Increased expression of pro-inflammatory cytokines, such as IL-1β and IL-6, have been reported in post-mortem brain tissue of patients with AD compared with non-demented controls (Griffin et al., 1989; Wood et al., 1993), and isolated microglia obtained from this tissue exhibit increased expression of these cytokines (Lue et al., 2001). Amyloid-beta (Aβ) has been shown to promote IL-1 production via activation of the inflammasome (Halle et al., 2008). Treatment of microglia with Aβ, especially in the presence of IFN-γ or LPS, promotes production of IL-1β, IL-6, and TNF-α (Gasic-Milenkovic et al., 2003; Meda et al., 1999). Furthermore, activated microglia expressing IL-1β and TNF-α are associated with Aβ plaques in sections taken from APP-transgenic mice (Benzing et al., 1999).

Activation of microglia is often accompanied by an increase in the expression of MHC class II, CD80, CD86, and CD40 (Aloisi et al., 2000b; Issazadeh, 1998), which is required for effective antigen presentation to T cells. The CNS was once thought to be an "immune privileged" site, isolated from the rest of the body by the blood–brain barrier (BBB), which minimises the passage of cells and macromolecules into the brain parenchyma. This understanding of the role of the adaptive immune system in the CNS was irrevocably altered with the discovery that activated T cells, regardless of their antigen specificity, have the potential to penetrate the intact BBB (Wekerle et al., 1987). It appears that antigen is initially presented to CNS-reactive T cells outside the CNS, and that these T cells subsequently cross the BBB, and are re-stimulated upon interaction with local APC, such as resident microglia, in the CNS. Microglia isolated from the CNS are capable of presenting antigen to Th1 cells, inducing production of IFN-γ and IL-2 (Aloisi et al., 2000b). It has also been shown that T cells can induce pro-inflammatory cytokine production from microglia (Chabot et al., 1997; Dasgupta et al., 2005).

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Studies with experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, have demonstrated that both Th1 and Th17 cells infiltrate the CNS, where they mediate autoimmune inflammation (Kebir et al., 2007; Kroenke et al., 2008; O'Connor et al., 2008). It has been suggested that Th1 cells promote macrophage activation, whereas Th17 cells recruit neutrophils into the CNS. However, the capacity of microglia to act as APC for Th1 and Th17 cells, and the modulatory effect of T cells on activation of microglia is not well characterised. IFN-γ, produced by Th1 cells, has been shown to induce pro-inflammatory cytokine production and co-stimulatory molecule expression by microglia (Meda et al., 1995; Nguyen and Benveniste, 2000; O'Keefe et al., 2002), and priming with IFN-γ appears to be required for Aβ-induced neuronal toxicity *in vitro* (Li et al., 2004).

The regulation of Th1 and Th17 cells in the CNS is not fully understood. Evidence from *in vitro* studies has suggested that IFN-γ and IL-4 may inhibit differentiation of Th17 cells (Park et al., 2005), whereas IL-10 and TGF-β have been implicated in controlling memory Th17 cells (Diveu et al., 2008; Mills, 2008; Rowan et al., 2008; Walsh et al., 2009). Th2 cells are generally considered to be anti-inflammatory, and together with regulatory T (Treg) cells, are considered to control inflammatory responses in the CNS as well as in the periphery (Battaglia et al., 2006; Moser and Murphy, 2000; Szczepanik et al., 2001). Indeed, the Th2-type cytokines, IL-4 and IL-10, have been found to attenuate Aβ-induced pro-inflammatory cytokine production by microglia (Szczepanik et al., 2001).

In this study we investigated the ability of Aβ-specific Th1, Th2, and Th17 cells to modulate Aβ-induced pro-inflammatory cytokine production and antigen presentation by microglia. We report that Aβ-specific Th1 or Th17 cells enhanced inflammatory cytokine production and MHC class II and co-stimulatory molecule expression on Aβ-treated microglia. Furthermore, we demonstrated that Th2 cells suppressed cytokine production by Th1 and Th17 cells, and attenuated activation of microglia by these T cells.

2. Materials and methods

2.1. Methods

2.1.1. Preparation of Aβ

Aβ (Biosource, USA) was dissolved in high-performance liquid chromatography-grade water to provide a 6 mg/ml stock solution, which was diluted to 1 mg/ml using sterile PBS, and allowed to aggregate for 48 h at 37 °C. Aβ was used immediately or stored at −20 °C until required. The presence of fibrillar Aβ was demonstrated by assessing the binding of thioflavin T (ThT, Sigma–Aldrich, UK). The endotoxin concentration in aggregated samples of Aβ was measured using the Pyrogene® Recombinant Factor C Endotoxin Detection System (Cambrex, USA), and preparations of Aβ used in these studies were found to contain less than 30 pg of endotoxin per 1 μg Aβ.

2.1.2. Preparation of primary glial cell cultures

Primary mixed glia were isolated from brains of postnatal 1-day old C57BL/6 mice as previously described (Downer et al., 2008). This preparation was chosen rather than purified microglia because we found that purified microglia have an activated phenotype with marked upregulation of CD40 and CD11b (Watson et al., unpublished). Mixed glia (2×10^6 cells/ml) were plated onto the centre of each well of a 6-well plate (250 μl per well) and allowed to adhere for 2 h at 37 °C in a humidified 5% CO_2 environment. Wells were then flooded with 1500 μl of pre-warmed DMEM. Media was changed every 2–3 days and glia were grown for 10–14 days before treatment. Using flow cytometry, we have established that these mixed glial cultures contain approximately 20% microglia and 80% astrocytes.

2.1.3. Treatment of cultured glia

All treatments were diluted to the required concentration in the appropriate pre-warmed, supplemented media and all solutions were filtered through a syringe with a cellulose acetate membrane filter (0.2 μM Supor membrane Acrodisc syringe filters, Pall Corporation, USA). Aβ (Biosource International, USA) was diluted to a final concentration (usually 40 μg/ml) in medium. Cells were treated with Aβ for 24 h. Recombinant IL-17 and IFN-γ (R&D Systems, USA) were reconstituted with PBS containing 1% bovine serum albumen (BSA) and diluted to a final concentration of 10 ng/ml in DMEM medium.

2.1.4. Generation of Aβ-specific T cell lines

C57BL/6 mice were immunised subcutaneously (s.c.) in the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse) and boosted 21 days later. After a further 7 days popliteal lymph nodes were harvested and isolated lymph node cells were re-stimulated *ex vivo* with Aβ (25 μg/ml) in the presence of polarising cytokines and antibodies as follows. Th1 cells were generated by addition of IL-12 (10 ng/ml) at the initiation of the culture. Th2 cell lines were generated by culture with dexamethasone (1×10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml) at the initiation of culture. Th17 cell lines were generated by culture with IL-1 (10 ng/ml), IL-23 (10 ng/ml), and anti-IFN-γ at the initiation of culture. After 4 days, IL-2 (5 ng/ml) was added to the Th1 and Th2 cell cultures. After a further 7 days, surviving T cells were washed and re-stimulated with antigen and splenic APC (irradiated spleen cells 2×10^6/ml) or used in co-culture experiments with mixed glia.

2.1.5. Co-culture of T cells and glia

Aβ-specific T cells were added to mixed glial cultures at a ratio of 0.5:1 (2.5×10^5 T cells:5×10^5 glia). After 24 h of culture supernatants were removed for cytokine analysis by ELISA, and for cell surface marker expression by flow cytometry.

2.1.6. Determination of cytokine concentrations by ELISA

The concentrations of IL-1β, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ and TNF-α in T cell and glial supernatants were measured by ELISA. Cytokine concentrations in the test samples were evaluated with reference to the standard curve prepared using recombinant cytokines of known concentration.

2.1.7. Flow cytometry

Cultured mixed glial cells were harvested and cells were washed and then blocked with 1 μg/ml of FcγR blocker (BD Biosciences, USA). Cells were stained with anti-MHC class II, anti-CD80, anti-CD86, or anti-CD40 antibody, or the appropriate isotype control antibody. For intracellular cytokine staining, Aβ-specific T cells were co-cultured with mixed glial cells for 24 h, and brefeldin A (5 μg/ml) was added for the last 12 h. Cells were harvested, washed, blocked, fixed, permeabilised (Intrastain kit, Dako, Denmark), and stained with anti-IL-17, anti-IFN-γ, anti-IL-6, or anti-TNF-α antibody, or the appropriate isotype control. Immunofluorescence analysis was performed using a Dako $CyAN_{ADP}$ flow cytometer (Dako, Denmark).

2.1.8. Statistical analysis

Statistical analysis was performed using the computer-based statistical package GraphPad Prism. One-way ANOVA using the Newman–Keuls post hoc test was used to determine statistically significant differences.

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3. Results

3.1. Generation of polarised Aβ-specific Th1, Th2, and Th17 cell lines

In order to examine the interaction between Aβ-specific T cell subsets and glia, we generated Aβ-specific Th1, Th2, and Th17 cell lines. Preliminary experiments showed that s.c. immunisation of mice in the footpad with Aβ and CpG induces primarily Th1 cells, and that the expansion of these cells *in vitro* could be enhanced by culture of the lymph node cells with antigen in the presence of IL-12. We found that we could generate a polarised Aβ-specific Th2 cell line by culturing lymph node cells from mice immunised with Aβ and CpG in the presence of IL-4 and glucocorticoids, which suppress IFN-γ production by $CD4^+$ T cells, while concomitantly enhancing IL-4-producing T cells (Li et al., 2007; Ramirez et al., 1996). We had previously reported that culture of spleen and lymph node from mice with EAE in the presence of MOG and IL-1β and IL-23, generates polarised MOG-specific Th17 cells (Sutton et al., 2006) and this approach was used to generate Aβ-specific Th17 cells.

T cell lines polarised under Th1-inducing conditions secreted high levels of IFN-γ, with low levels of IL-4, IL-10, and IL-17 (Fig. 1A). Th2-polarised T cells produced low levels of IFN-γ, IL-10, and IL-17. IL-4 could not be accurately measured in supernatants from Th2-polarised cells at this point, due to the addition of exogenous IL-4. The Th17 cell line produced high levels of IL-17, with no IFN-γ, IL-4, IL-10, and small amounts of IL-5 (Fig. 1A).

Restimulation of Th17 cells through their T cell receptor (TCR) has been shown to induce their conversion to Th1 cells by increasing T-bet expression (Mathur et al., 2006). To determine whether our protocols induced stably-polarised Th1, Th2, and Th17 cell lines, Aβ-specific T cell lines were re-stimulated with Aβ and irradiated splenic APC in the absence of polarising cytokines. Following antigen-restimulation, the Th1 cell line produced high concentrations of IFN-γ, and low concentrations of IL-4, IL-10, and IL-17 (Fig. 1B). The Th2 cell line secreted high levels of IL-4

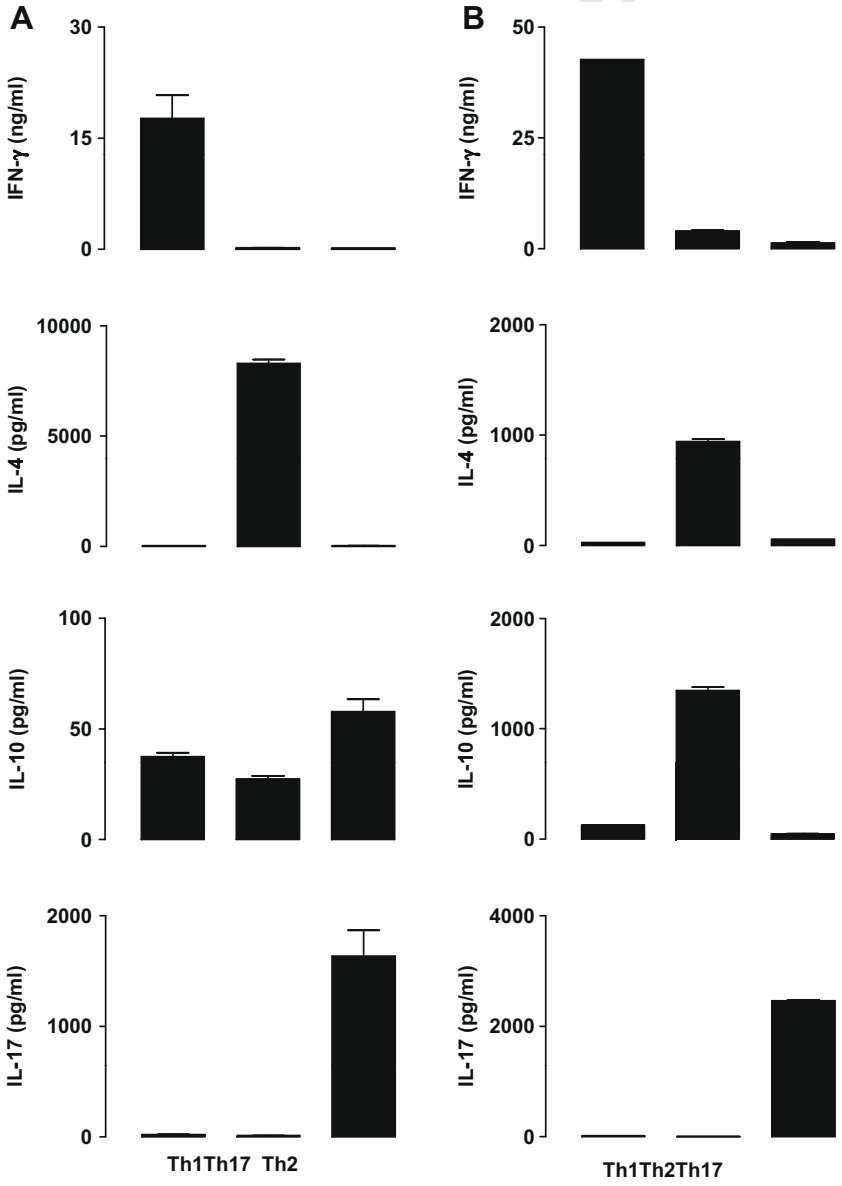

Fig. 1. Cytokine production by Aβ-specific Th1, Th2, and Th17 cell lines. Short term Aβ-specific $CD4^+$ T cell lines were generated from mice immunised with CpG and AB by stimulation of spleen cells with antigen under Th1, Th2, or Th17 polarising conditions. The production of IFN-γ, IL-4, IL-10, and IL-17 by the Aβ-specific Th1, Th2, and Th17 cell lines was tested by stimulation with Aβ and splenic APC 4 days after one (A) and two (B) rounds of antigen-stimulation.

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and IL-10, and low levels of IFN-γ and IL-17. Th17 cell lines produced large amounts of IL-17, and low levels of IFN-γ, IL-4, and IL-10 (Fig. 1B). These data indicate that this method of polarisation induced homogenous Aβ-specific Th1, Th2, and Th17 cell lines.

3.2. Aβ-specific Th2 cells inhibit IFN-γ and IL-17 production by Th1 and Th17 cells respectively

Previous studies have shown that microglia are capable of acting as an APC to induce proliferation and IFN-γ and IL-12 production by Th1 cells *in vitro* (Aloisi et al., 2000a; Becher et al., 2000). However the capacity of glia to act as APC for Th17 cells has not been addressed. Here, we examined the capacity of glia to induce the production of cognate cytokines from Th1 and Th17 cell lines. Aβ-specific Th1 or Th17 cells were cultured with mixed glia and Aβ, and IFN-γ and IL-17 production was determined by intracellular cytokine staining using flow cytometry. Gating on $CD4^+$ T cells also allowed us to confirm that the cytokine-secreting cells were true Th1 and Th17 cells. The data show that co-culture of glia and Th1 cells induced T cell production of IFN-γ, with little IL-17, while Th17 cells co-cultured with glia produced significant levels of IL-17 and no IFN-γ (Fig. 2A). It has been reported that IL-4 can suppress differentiation of Th17 cells from naïve T cells (Harrington et al., 2005). Th2 cells can also inhibit Th1 responses. Here, we examined the influence of Aβ-specific Th2 cells on microglia and antigen-induced activation of Aβ-specific memory Th1 and Th17 cells. Addition of Th2 cells attenuated IFN-γ production by Th1 cells and IL-17 production by Th17 cells (Fig. 2A). Our findings demonstrate that glia act as an effective APC for antigen-induced cytokine production by memory Th1 and Th17 cells, and that this is regulated by antigen-specific Th2 cells. We also examined IL-4 production by the different T cell populations stimulated with Aβ and mixed glia. The data show that Th2, but not the Th1 and Th17 cells, secrete IL-4 in response to activation with Aβ using mixed glia as the APC (Fig. 2B). The reduction in IL-4 concentration in the presence of Th1 or Th17 cells may reflect consumption of the cytokine from the cultures by binding to IL-4 receptors expressed on Th1 and Th17 cells. This possibility is consistent with the fact that IL-4 is known to regulate Th1 and Th17 cells (Glimcher and Murphy, 2000; Moser and Murphy, 2000; Park et al., 2005).

3.3. Aβ-specific Th1 and Th17 cells induce glial pro-inflammatory cytokine production and co-stimulatory molecule expression

Previous studies have shown that interaction of microglial cells with antigen-specific T cells results in the upregulation of co-stimulatory molecules and production of pro-inflammatory cytokines (Aloisi et al., 2000a; Chabot et al., 1997; Dasgupta et al., 2005). To assess the comparative effect of Aβ-specific Th1 and Th17 cells on microglial activation, polarised Th1 and Th17 cells were co-cultured with Aβ-treated mixed glia. Aβ-induced significant production of IL-6 and TNF-α from mixed glia ($F_{(3,16)} = 187.5$, $p < 0.05$; $F_{(3,16)} = 68.35$, $p < 0.05$; Fig. 3A). Treatment of glia with Aβ-specific Th1 or Th17 cells enhanced Aβ-induced IL-1β ($F_{(3,16)} = 22.04$, $p < 0.05$ and $p < 0.001$, respectively), IL-6 ($F_{(3,16)} = 187.5$, $p < 0.001$ and $p < 0.001$, respectively), and TNF-α ($F_{(3,16)} = 68.35$, $p < 0.001$ and $p < 0.001$, respectively) production. In addition, supernatants from co-cultures of glia and Th17 cells had significantly higher IL-1β ($F_{(3,16)} = 22.04$, $p < 0.01$) and IL-6 ($F_{(3,16)} = 187.5$, $p < 0.001$) levels than glia co-cultured with Th1 cells, suggesting that Aβ-spe-

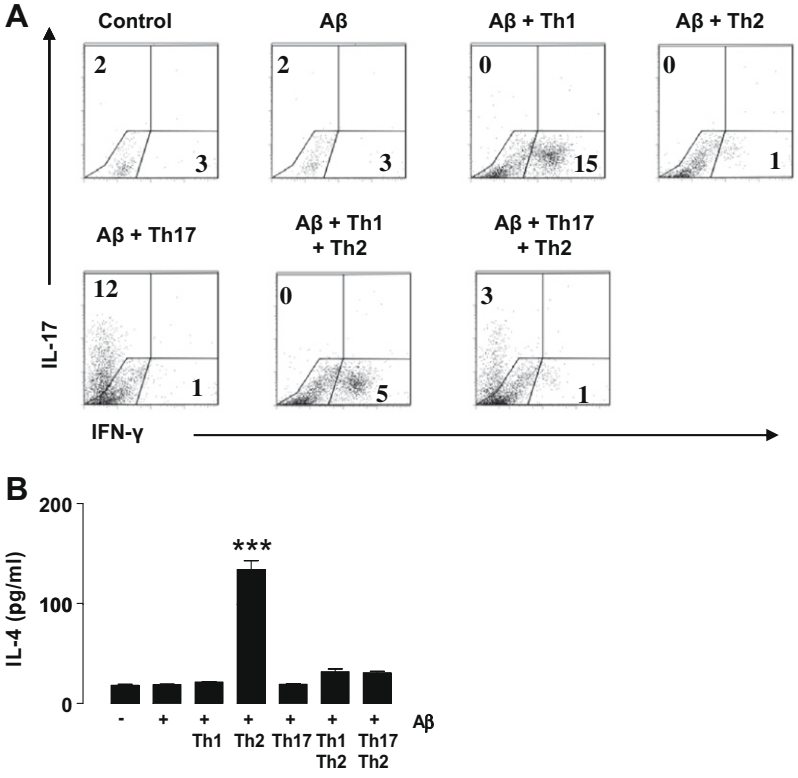

Fig. 2. Glia act as APC for Aβ-specific Th1 and Th17 cells, promoting IFN-γ and IL-17 production, and this is inhibited by Th2 cells. C57BL/6 mice were injected s.c. in the footpad with Aβ (75 μg/mouse) and CpG (25 μg/mouse), and boosted 21 days later. Popliteal lymph nodes were harvested after a further 7 days and cells were re-stimulated with Aβ under Th1, Th2 or Th17 polarising conditions. Mixed glia from C57BL/6 mice were cultured with Aβ (40 μg/ml) alone or with Aβ-specific Th1 or Th17 cells, in the presence or absence of Aβ-specific Th2 cells. (A) After 12 h brefeldin A (5 μg/ml) was added. After a further 12 h, the percentage of $CD4^+$ T cells expressing IFN-γ and IL-17 was determined by flow cytometry. Dotplots of IFN-γ vs IL-17 expression are shown from each treatment group. (B) Concentrations of IL-4 in supernatants were quantified by ELISA.

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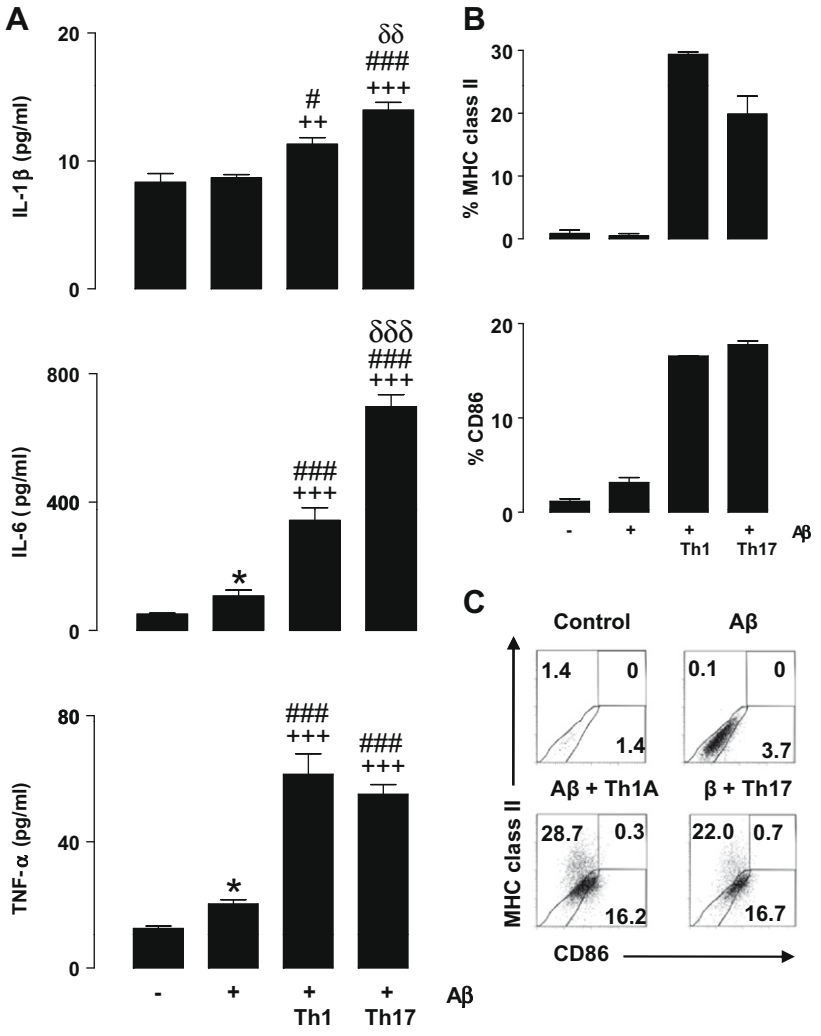

Fig. 3. Aβ-specific Th1 and Th17 cells promote pro-inflammatory cytokine production and co-stimulatory molecule expression on microglia. Mixed glial cultures from C57BL/6 mice were cultured with Aβ (40 μg/ml) alone or with Aβ-specific Th1 or Th17 cell lines. (A) After 24 h, concentrations of IL-1β, IL-6, and TNF-α in supernatants were quantified by ELISA. (B) Expressing MHC class II, or CD86 on $CD11b^+$ cells was determined by FACS analysis and expressed as a percentage of the total population of cells expressing CD11b. (C) Representative dotplots are shown from each treatment group. $^{*}p < 0.05$, Aβ vs medium-treated control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, Aβ vs Aβ + Th1/Th17; $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$, Aβ + Th17 vs Aβ + Th1; ANOVA.

cific Th17 cells are more potent inducers of glial activation than Th1 cells (Fig. 3A).

The effect of Th1 and Th17 cells on the expression of co-stimulatory molecules on the surface of microglia was examined by flow cytometry. Co-culture of glia and Th1 or Th17 cells enhanced Aβ-induced MHC class II and CD86 expression on microglia (Fig. 3B and C). Collectively our findings suggest that Th1 and Th17 are capable of promoting both inflammatory cytokine production and MHC and co-stimulatory molecule expression on microglia.

3.4. IFN-γ and IL-17 enhance Aβ-induced activation of microglia

The data presented above demonstrate that co-culture of Aβ-specific Th1 or Th17 cells and microglia induces pro-inflammatory cytokine production and APC capacity of microglia. We next examined the capacity of IFN-γ and IL-17, the signature cytokines of Th1 and Th17 cells, to enhance Aβ-induced glial activation. Treatment with IFN-γ or IL-17 alone did not induce pro-inflammatory cytokine production by mixed glia (Fig. 4A). However, IL-17 was found to enhance IL-6 production from Aβ-stimulated glia ($F_{(5,47)} = 63.41$, $p < 0.001$), while treatment with IFN-γ in combination with Aβ-induced significantly greater production of IL-6 ($F_{(5,47)} = 64.24$, $p < 0.001$) and TNF-α ($F_{(5,47)} = 64.24$, $p < 0.001$) from mixed glia than treatment with either Aβ or IFN-γ alone (Fig. 4A).

Analysis of MHC class II and co-stimulatory molecule expression on the surface of microglia revealed that IFN-γ induced MHC class II, CD86, and CD40 expression on $CD11b^+$ microglia, (Fig. 4B and C). IFN-γ also enhanced Aβ-induced CD86 and CD40 expression. IL-17 had no effect on MHC class II or co-stimulatory molecule expression on microglia either alone or in combination with Aβ. These data indicate that Aβ-induced microglial activation is enhanced by Th1 and Th17 cells, and that IFN-γ is partly responsible for Th1 cell-mediated activation of microglia. However, activation of microglia by Th17 cells appears to require Th17-derived factors other than IL-17, or may be dependent on cell–cell interactions between the microglia and the T cell.

3.5. Aβ-specific Th2 cells attenuate pro-inflammatory activation of microglia by Th17 cells, but not by Th1 cells

Having shown that Th1 and Th17 cells enhance Aβ-induced microglial activation and that Aβ-specific Th2 cells inhibit cytokine production by Th1 and Th17 cells in response to stimulation with

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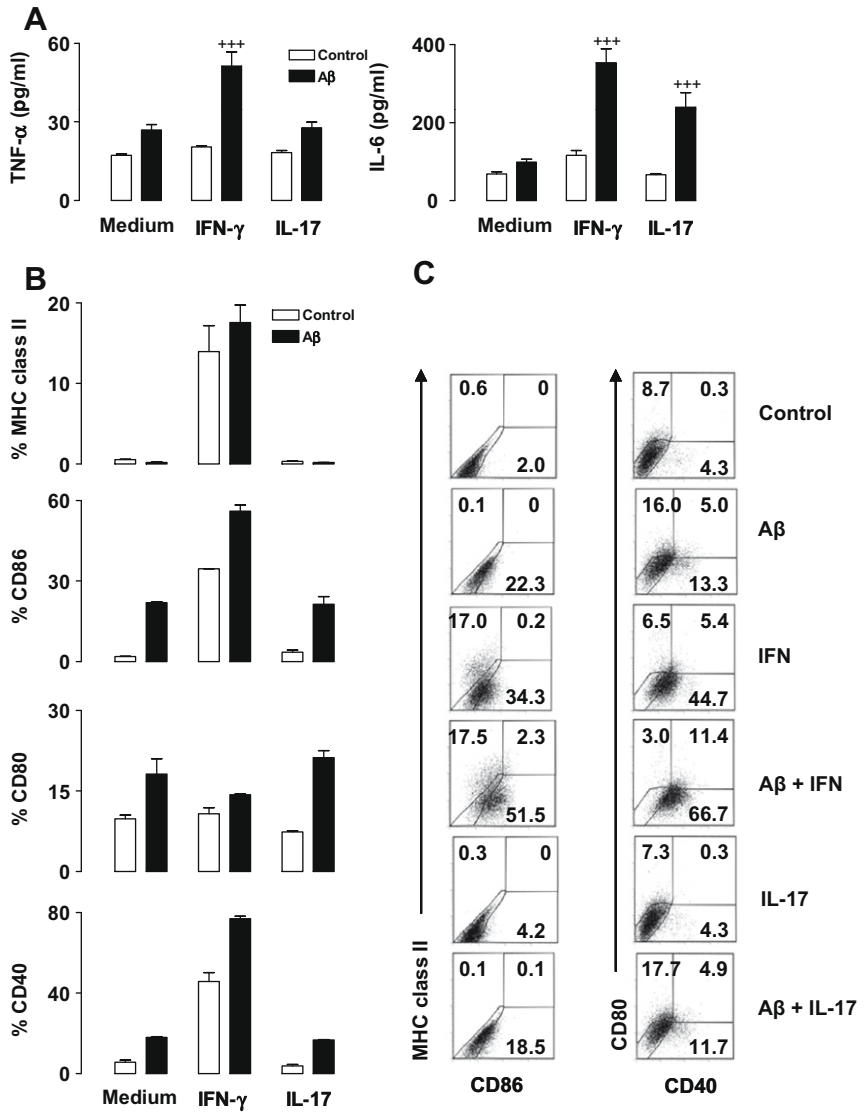

Fig. 4. IFN-γ and IL-17 enhance Aβ-induced activation of microglia. Mixed glia from 0 to 1 day old C57BL/6 mice were cultured with Aβ (40 μg/ml) in the presence or absence of IFN-γ (10 ng/ml) or IL-17 (10 ng/ml). (A) After 24 h, concentrations of IL-6 and TNF-α in supernatants were measured by ELISA. (B) Expressions of MHC class II, CD86, CD80, or CD40 on $CD11b^+$ cells were determined by FACS analysis and expressed as a percentage of the total population of cells expressing CD11b. (C) Representative dotplots are shown from each treatment group. $^{+++}p < 0.001$, Aβ vs Aβ + IFN-γ or Aβ + IL-17; ANOVA.

Aβ and microglia as APC, we examined the capacity of Th2 cells to suppress cytokine production and co-stimulatory molecule expression induced by co-culture of mixed glia and Th1 or Th17 cells. Th2 cells alone slightly attenuated Aβ-induced TNF-α production ($p < 0.05$), but had no significant effect on Aβ-induced IL-1β or IL-6 production by mixed glia. Addition of Th2 cells to co-cultures of glia and Th17 cells significantly inhibited the production of IL-1β ($F_{(6,34)} = 10.28$, $p < 0.001$) and IL-6 ($F_{(6,32)} = 12.19$, $p < 0.001$; Fig. 5A). Addition of Th2 cells did not reduce production of IL-6 induced by co-culture of glia and Th1 cells. Th2 cells had no effect on increases in TNF-α production induced by co-culture of glia and Th1 cells.

Increases in cytokine production seen following co-culture of glia and Aβ-specific T cells may be due to the production of these cytokines by either the glia or the T cells. To determine the contribution of T cells to the pro-inflammatory cytokine production seen following co-culture, cytokine production by T cells was assessed by intracellular cytokine staining. It was found that only a small percentage of T cells produced IL-6 and TNF-α in response to co-culture with Aβ-stimulated glia, indicating that they are unlikely to contribute significantly to the pro-inflammatory cytokine production seen in co-culture of T cells with mixed glia (Fig. 5B).

The effect of Aβ-specific T cell subsets on the expression of co-stimulatory molecules on the surface of glia was examined by flow cytometry. It was found that co-culture of mixed glia and either Th1 or Th17 cells enhanced Aβ-induced MHC class II, CD80, CD86, and CD40 expression on $CD11b^+$ microglia (Fig. 6A). Addition of Th2 cells attenuated Th1 cell-induced CD86 and CD40 expression on microglia, while enhancing Th1 cell-induced CD80 and MHC class II expression. Th17 cell-induced CD80 and MHC class II expression on microglia was enhanced by addition of Th2 cells, while CD86 expression on microglia was attenuated by Th2 cells (Fig. 6A). Flow cytometry analysis of the $CD11b^-$ cells (predominantly astrocytes) within the mixed glial population revealed that these cells express lower basal expression of MHC class II and co-stimulatory molecules. This was only marginally enhanced by culture with Th1 or Th17 cells, and was not reduced by co-culture with Th2 cells (Fig. 6B). The data show that Aβ-specific Th2 cells can inhibit pro-inflammatory cytokine production induced by co-culture of mixed glia and Th17, but not Th1 cells. In addition,

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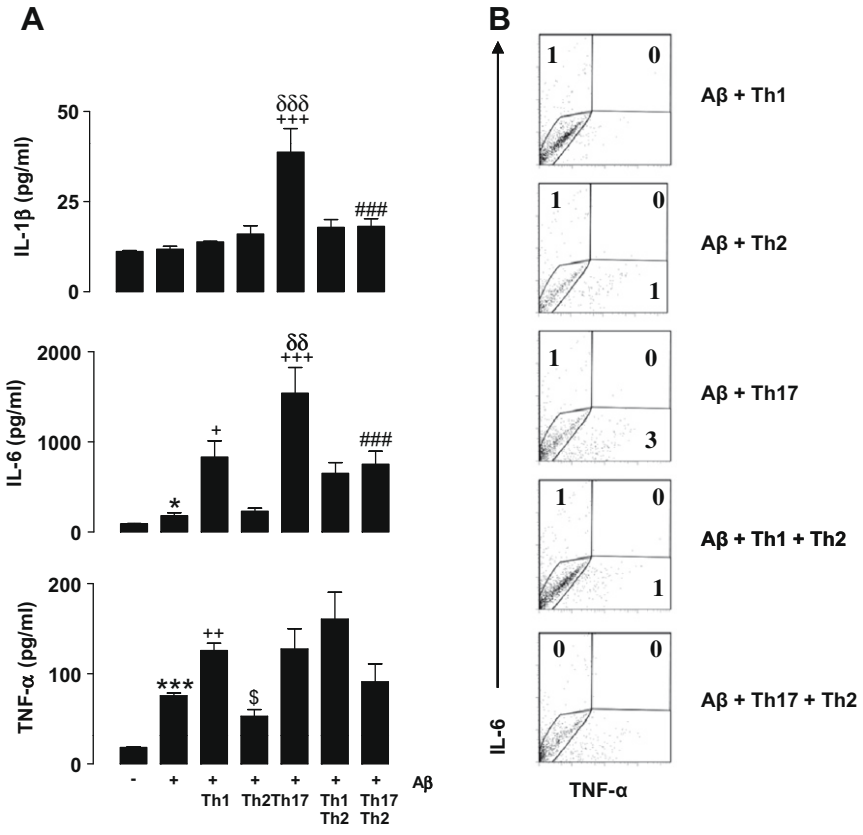

Fig. 5. Aβ-specific Th2 cells attenuate pro-inflammatory cytokine production by Th17 cells, but not by Th1 cells. Mixed glial cultures from C57BL/6 mice were cultured with Aβ (40 μg/ml) alone or with Aβ-specific Th1, Th2, or Th17 cell lines. (A) After 24 h, concentrations of IL-1β, IL-6, and TNF-α in supernatants were measured by ELISA. (B) Brefeldin A (5 μg/ml) was added after 12 h or culture, and percentage of $CD4^+$ T cells expressing IL-6 and TNF-α was determined by intracellular staining using flow cytometry. $^{*}p < 0.05$, $^{***}p < 0.001$, Aβ vs medium-treated control; $^{+}p < 0.05$, $^{++}p < 0.01$, Aβ vs Aβ + Th1; $^{+++}p < 0.001$, Aβ vs Aβ + Th17; $^{\$}p < 0.05$, Aβ vs Aβ + Th2; by ANOVA.

Th2 cells have distinct modulatory effects on Th1 and Th17 cell-induced co-stimulatory molecule expression on microglia, but appear to have little effect on astrocytes.

4. Discussion

The results presented in this study demonstrate that glia act as an effective APC for Aβ-specific Th1 and Th17 cells, which in turn can enhance Aβ-induced pro-inflammatory cytokine production and co-stimulatory molecule expression on microglia. In addition, Th2 cells were found to suppress glial induction of IFN-γ and IL-17 production by Th1 and Th17 cells respectively, and attenuate activation of microglia by Aβ-specific Th17 or Th1 cells.

The differentiation of naive T cells into distinct T cell subtypes and the expansion of memory CD4 T cells are predominantly determined by the cytokine environment present at the site of activation (Glimcher and Murphy, 2000; Mosmann et al., 1986). Consistent with previous reports on the role of IL-12 and IL-4 in promoting Th1 and Th2 differentiation with conventional APC (Gately et al., 1998; Rao and Avni, 2000), we found that culture with antigen in the presence or IL-12 or IL-4 selectively expanded Aβ-specific Th1 and Th2 cells, respectively from mice immunised with Aβ and CpG. However, the Th2 cells lines were more polarised following antigen-stimulation with a combination of dexamethasone and IL-4. Dexamethasone is reported to induce apoptosis which is rescued by IL-4 (Zubiaga et al., 1992), allowing selective survival and expansion of Th2 cells. A combination of IL-1 and IL-23, but not IL-23 alone (data not shown), was found to be capable of selectively expanding Th17 cells. This is supported by our previous study which demonstrated an essential role for IL-1 in the development of MOG-specific Th17 cells that are pathogenic in EAE (Sutton et al., 2006).

This study demonstrated that mixed glia acted as an effective APC for Aβ-specific T cells, inducing IFN-γ and IL-17 production by Th1 and Th17 cells, respectively. Since we used mixed glia, we cannot be certain that microglia are the APC population presenting antigen to the T cells. Indeed, astrocytes in mixed glial cultures have been shown to be capable of expressing MHC class II, and they can also present myelin basic protein (MBP) to encephalitogenic T cell lines (Dong and Benveniste, 2001; Fontana et al., 1984). However, in the co-cultures of mixed glia with T cells, we observed that the intensity of expression and percentage cells expressing MHC class II, CD80 and CD86 was substantially higher on microglia than on astrocytes. Even allowing for the greater number of astrocytes in the mixed glia culture, it would appear that microglia are the major population of APC in our system. Nevertheless, we do not rule out a role for astrocytes, either in antigen presentation or suppression of immune responses. Although we did not make a direct comparison, our studies suggest that splenic APC and mixed glia were both efficient APC for Aβ-specific Th1, Th2 and Th17 cells. It has been reported that microglia can induce IFN-γ production from ovalbumin-specific Th1 cells (Aloisi et al., 1998). However, a number of studies have reported that microglia induce T cell activation only in the presence of IFN-γ (Aloisi et al., 1998; Li et al., 2004; Matsumoto et al., 1992). It has also been reported that brain dendritic cells (DC) from mice injected with IFN-γ support IL-17 production by naïve OVA-specific T cells *in vitro*, whereas microglia or splenic DC were ineffective at inducing IL-17 production, but did support proliferation and IFN-γ production (Gottfried-Blackmore et al., 2009). The discrepancy between the published results

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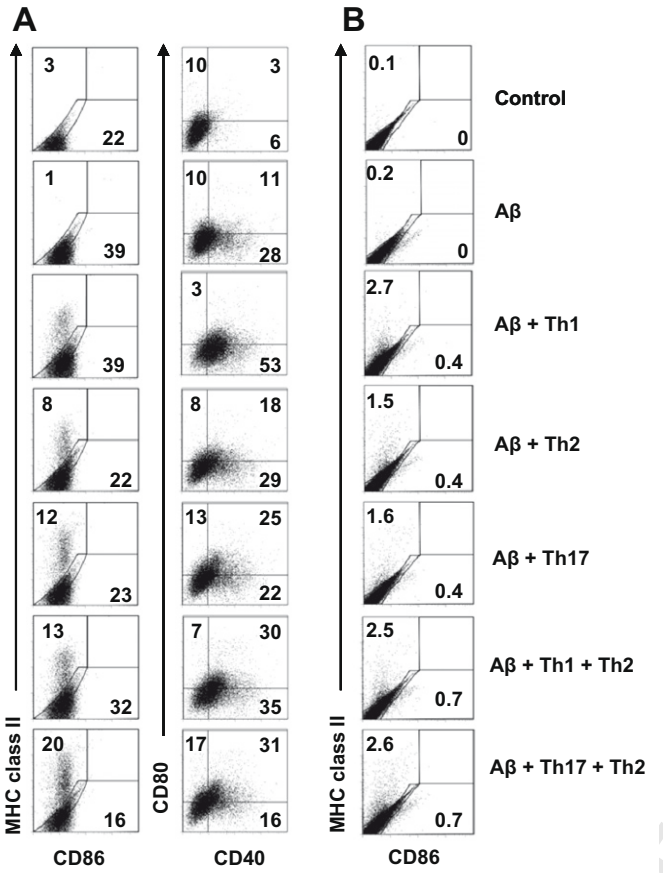

Fig. 6. Distinct modulatory effects of Aβ-specific Th2 cells on Th1 and Th17 cell-induced co-stimulatory molecule expression on microglia, but not astrocytes. Mixed glial cultures from C57BL/6 mice were treated with Aβ (40 μg/ml) alone or with Aβ-specific Th1, Th2, or Th17 cell lines. After 24 h, expression of MHC class II, CD86, CD80, or CD40 on $CD11b^+$ cells (A) or $CD11b^-$ (B) were quantified by FACS analysis and expressed as a percentage of the total population of cells expressing CD11b. Dotplots of MHC class II vs CD86 expression, and CD80 vs CD40 expression on $CD11b^+$ cells (A), or MHC class II vs CD86 expression on $CD11b^-$ cells (B) are shown from each treatment group.

and those in the present study may reflect the fact that we used mixed glia as APC, or that Aβ is an autoantigen, but also acts as an innate inflammatory stimulus. Aβ has been shown to promote IL-1β production in combination with TLR agonists through activation of the inflammasome and caspase-1 (Halle et al., 2008). We and others have also shown that Aβ can induce the production of co-stimulatory molecules from glia (Lyons et al., 2007), and it has been suggested that Aβ may act as both antigen and co-stimulator to promote innate and adaptive immune response (Loewenbrueck et al., 2008).

A major host protective function of Th1 and Th17 cells is to promote adaptive cellular immunity to infection. However, these cells types also promote inflammation and are central to the pathology in many chronic inflammatory and autoimmune conditions, including CNS inflammation in EAE and MS. The present study shows that both Th1 and Th17 cells are capable of promoting activation of microglia in response to Aβ. Previous studies have shown that Aβ induces pro-inflammatory cytokine production from mixed glial cultures *in vitro* (Floden and Combs, 2006; Ii et al., 1996; Murphy et al., 1998), and it is thought that microglia are the main source of pro-inflammatory cytokines in the CNS (Benveniste, 1992). In the present study, Aβ-induced production of the pro-inflammatory cytokines TNF-α and IL-6 by mixed glia, which was significantly enhanced by and co-culture with Th1 or Th17 cells. Interestingly, Aβ-specific Th17 cells induced greater production of IL-1β and IL-6 by glia than Aβ-specific Th1 cells. Th1 cells specific for ovalbumin have been shown to promote MHC class II and CD40 expression on the microglia (Aloisi et al., 1998). Here, we demonstrate that both Th17 and Th1 cells specific for Aβ are capable of enhancing expression of MHC class II, CD80, CD86, and CD40 on Aβ-activated microglia. This is the first report showing that Th17 cells can activate microglia and suggests that this may be one mechanism whereby this T cell subset mediates its inflammatory effect in the CNS.

In the context of AD, CD40–CD40L interaction appears to be important in Aβ-induced microglial activation and in pathology; APP-transgenic mice lacking CD40L have decreased astrocytosis and microgliosis and this is associated with diminished Aβ plaque burden (Tan et al., 2002). Furthermore, CD40L is expressed on astrocytes as well as on T cells and this is enhanced in AD (Calingasan et al., 2002). Therefore, it is possible that microglial activation induced by Th1 and Th17 cells is mediated indirectly by enhancing CD40L expression on astrocytes, which in turn interact with CD40 expressed on the microglia.

In an attempt to define the role of Th1- and Th17-derived cytokines in microglial activation, we examined the direct effect of the signature cytokines IL-17 and IFN-γ. Consistent with previous studies which reported that IFN-γ enhanced Aβ-induced TNF-α and nitric oxide production by glia (Goodwin et al., 1995; Meda et al., 1995), we found that Aβ and IFN-γ synergised in triggering production of IL-6 and TNF-α, as well as expression of CD86 and CD40. In contrast, while Th17 cells enhanced pro-inflammatory cytokine and co-stimulatory molecule expression on glia, IL-17 only enhanced IL-6 and had no effect on TNF-α production, or expression of MHC class II or CD86 on microglia. Therefore, it is possible that Th17 may mediate its effect via other cytokines or may require direct interaction with the glia.

Although Treg cells are considered to be centrally involved in the control of inflammatory T cells, Th2 cells can cross regulate Th1 responses in the periphery (Glimcher and Murphy, 2000; Moser and Murphy, 2000). Furthermore, IL-4 has a major anti-inflammatory function in the brain (Lyons et al., 2007). IFN-γ and IL-12 expression are enhanced and IL-4 reduced in the cerebral cortex of APP-transgenic mice (Abbas et al., 2002). In addition, Aβ-specific Th2 cells have been shown to reduce plaque-associated microglia and improve cognition in APP/PS1 transgenic mice (Cao et al., 2009). In the present study, we found that Aβ-specific Th2 cells did not induce IL-1β, TNF-α, or IL-6 production when co-cultured with glia, but attenuated the production of IL-1β and IL-6 in co-cultures of glia and Aβ-specific Th17 cells. This may in part be mediated by the inhibitory effect of Aβ-specific Th2 cells on IL-17 production by Th17 cells. The Th2-type cytokines, IL-4 and IL-10, have previously been shown to reduce microglial pro-inflammatory cytokine production induced by LPS and IFN-γ (Chao et al., 1993; Ledeboer et al., 2002; Nguyen and Benveniste, 2000; Sawada et al., 1999). Despite producing large amounts of IL-4 however, Th2 cells were unable to attenuate pro-inflammatory cytokine production induced by co-culture of glia and Th1 cells. However, Th1 cell-induced IFN-γ production was found to be inhibited by Th2 cells, suggesting that production of pro-inflammatory cytokines in co-cultures of glia and Th1 cells is predominantly cell-contact dependent. Th2 cells were also found to modulate co-stimulatory molecule expression on microglia induced by Th1 and Th17 cells. Addition of Th2 cells increased both Th1 and Th17 cell-induced MHC class II and CD80, while reducing Th1 and Th17 cell-induced CD86. Th2 cells were also found to decrease Th1 cell-induced CD40. It has previously been reported that CD40:CD154 interaction is important in the interaction of microglia and T cells (Laporte et al., 2006; Tan et al., 2002; Townsend et al., 2005), and Nguyen and colleagues have found that IL-4 inhibits IFN-γ-induced CD40

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(Nguyen and Benveniste, 2000). However, inhibition of CD40 by Th2 cells did not affect their induction of pro-inflammatory cytokine production by co-cultures of glia and Th1 cells.

Activation of microglia is known to be regulated by interaction with other cell types in the brain. Indeed, studies from this and other laboratories have shown that expression of CD200 and fractalkine by neurons can attenuate microglial activation (Hoek et al., 2000; Lyons et al., 2009a,b). It is therefore possible that some of the modulatory effects of T cells on microglial activation are mediated through their effects on astrocytes. Although astrocytes are a poor APC for Th1 cells, they are an effective APC for Th2 cells (Aloisi et al., 1998) and Treg cells (Trajkovic et al., 2004). As Th2 cells are known to inhibit both Th1 and Th17 responses, it is possible that Th2 cells inhibit Th17 responses in our system through interaction with astrocytes. Alternatively, astrocyte-activated Th2 or Treg cells could inhibit Th1 and Th17 cell responses through the induction soluble factors, such as the anti-inflammatory cytokines IL-4, IL-10 or TGF-β, or through a cell-contact dependant mechanism.

We have shown that Th17 cells are potent activators of microglia, enhancing expression of MHC class II and CD86 and inducing production of IL-1β, IL-6 and TNF-α in co-culture with mixed glia. This Th17 cell-induced activation of microglia was found to be suppressed by Th2 cells. However, we also found that, while recombinant IL-17A promoted IL-6 production by mixed glia, it had little effect on CD40, CD80 or CD86 expression. Th17 cells may mediate at least some of their effects through cytokines other than IL-17A, such as IL-17F, IL-21 and IL-22, which are also secreted by this T cell subtype and have distinct functions in inflammatory responses (Mills, 2008). Therefore the inhibitory effects of Th2 cells on Th17-induced microglial activation may involve inhibition of soluble factors other than IL-17.

We also found that Th1 cells are potent activators of microglia. Here, IFN-γ, like the Th1 cells, had a more profound activating effect on microglia, significantly enhancing MHC class II, CD40 and CD86 expression and production of TNF-α. Thus it appears that the IFN-γ, the signature cytokine of Th1 cells is a major mediator of effect of Th1 cell-induced microglial activation. While Th2 cells did not suppress Th1-induced cytokine production by mixed glia, they did suppress CD40 and CD86 expression. Therefore, since Th2 cells inhibit IFN-γ production by Aβ-specific Th1 cells, this may be the mechanism of suppression by Th2 cells of this aspect of Th1 cell-induced activation of microglia. Alternatively, it is possible that inhibition of Th1 and Th17 responses by Th2 cells could be mediated through a cell–cell-contact mechanism. Although we do not have data to support this hypothesis, it has been demonstrated that Treg cells suppress effector T cells through direct contact with the target T cell or the APC, as well as through the secretion of immunosuppressive cytokine (Vignali et al., 2008).

While acknowledging the limitations of *in vitro* experiments with cultured glia, which may not necessarily reflect their function *in vivo*, we conclude that Th1 and Th17 cells activate microglia to an inflammatory phenotype, characterised by pro-inflammatory cytokine production and co-stimulatory molecule expression.

In contrast to the effects of Th17 and Th1, Th2 cells did not induce production of IL-1β, IL-6, or TNF-α, and did not affect co-stimulatory molecule expression by microglia, but they were able to inhibit the activation of glia induced by Th17 cells. These findings suggest that T cells have a profound effect on the Aβ-induced microglial activation and suggest that selective induction of Aβ-specific T cell subtypes may be a useful approach to modulate inflammatory responses to Aβ in the CNS.

This study provides new insight into the role of Aβ-specific T cells and their associated cytokines in microglial activation. Our findings also have implications for our understanding of the potential role of T cell subtypes in the pathogenesis of AD. Activated T cells are capable of crossing the intact BBB, and infiltrating Th1 and Th17 cells have both been shown to play distinct role in promoting the inflammatory pathology in experimental autoimmune encephalomyelitis, whereas Th2 and Treg cells are protective (Kebir et al., 2007; Kroenke et al., 2008; Mills, 2008; O'Connor et al., 2008; Park et al., 2005; Rowan et al., 2008; Walsh et al., 2009). Although there is no clear evidence of T cell responses in the brains of AD patients, a number of AD patients developed meningoencephalitis following immunisation with Aβ in adjuvant and this was thought to be mediated by Th1 cells specific for Aβ (Boche and Nicoll, 2008; Holmes et al., 2008). While it is still not clear whether inflammation in the CNS precipitates, or is a consequence of the neurodegenerative changes associated with the development of AD, our data point to a potentially pathogenic role for Th1 and Th17 cells and protective role for Th2 cells in CNS inflammation.

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