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Treatment with chimeric anti-human CD40 antibody suppresses MRI-detectable inflammation and enlargement of pre-existing brain lesions in common marmosets affected by MOG-induced EAE

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Abstract

Common marmosets, a Neotropical monkey species, are protected against clinical and neuropathological consequences of experimentally induced autoimmune encephalomyelitis (EAE) by prophylactic treatment with ch5D12, a humanized antagonist antibody against human CD40. In the current study we have tested whether ch5D12 acts therapeutically against the enlargement and inflammatory activity of existing (brain) white matter lesions using serial magnetic resonance imaging (MRI). The results show in all PBS treated monkeys ($n=4$) a rapid enlargement of T2 lesions together with an increment of the T2 signal intensity due to inflammatory edema. Treatment with ch5D12 delayed the enlargement of T2 lesions in 2 out of 3 tested monkeys while in 3 out of 3 monkeys the T2 signal increment of lesions was suppressed. In conjunction with previously published data on the clinical benefit of anti-CD40 treatment in the marmoset EAE model, the current findings support antibody-mediated blockade of CD40 interaction with its ligand CD154 as a potential treatment of MS.

Keywords: EAE; CD40; Antibody therapy; Non-human primate

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) in the common marmoset (*Callithrix jacchus*) is a useful preclinical model of multiple sclerosis (reviewed in (Brok et al., 2001; Genain and Hauser, 2001; 't Hart et al., 2000, 2004b)). The central nervous system (CNS) white matter lesions that develop in the various versions of this EAE model share pathomorphological, radiological and immunological features with MS (Laman et al., 1998b; Raine et al., 1999; 't Hart et al., 1998). Hence, the marmoset EAE model can bridge the wide immunological gap between humans and rodents that hampers the selection of promising treatments in the drug development pipeline at a preclinical stage (Mestas and Hughes, 2004; Sachs, 2003; 't Hart et al., 2004a).

Marmosets immunized with rhMOG, a recombinant protein representing the extracellular fragment of human MOG (amino acids 1–125) develop EAE in 100% of the cases, which is due to the presence of the monomorphic MHC class II susceptibility element Caja-DRB*W1201 in the repertoire of each monkey (Antunes et al., 1998; Bontrop et al., 1999; Brok et al., 2000; Villoslada et al.,

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2001). A particularly useful aspect of this model for therapy development is that lesions developing in the brain white matter can be visualized and tentatively characterized with clinically relevant magnetic resonance imaging techniques (Jordan et al., 1999; 't Hart et al., 2004c). Longitudinal analysis of the brain white matter lesions with magnetic resonance imaging (MRI) showed a progressive increment of the volume and persistent inflammatory activity in the majority of the lesions. Furthermore, the characterization of the CNS pathology with MRI and previously described histological criteria ('t Hart et al., 1998) revealed that the majority of the lesions are in an early active stage ('t Hart et al., 2004d).

We have used the rhMOG-induced EAE model to test whether antibodies targeting co-stimulatory molecules of antigen presenting cells (APC) and T-cells are a potential treatment for MS. The interaction of CD40 with its ligand CD154 plays an important role in various immunopathogenic processes that operate in EAE, including B-cell activation, antigen-presenting cell (APC) activation, initiation of antigen-specific T-cell responses and induction of macrophage effector functions (Grewal and Flavell, 1996; Laman et al., 1996; Quezada et al., 2004; van Kooten and Banchereau, 1997). Our initial studies confirmed that mice treated with an antibody against CD154 are protected against EAE (Gerritse et al., 1996). However, a clinical trial in MS patients with antibody against CD154 was stopped due to unexpected side-effects that were not observed in the animal experiments (Kawai et al., 2000).

The mouse monoclonal antibody (Mab) 5D12 (mu5D12) has been raised against human CD40. The 5D12 antibody appeared a potent inhibitor of CD40–CD40L mediated activation on several cell types and, unlike most other anti-CD40 Mabs, does not exert CD40 stimulatory activity (Kwekkeboom et al., 1993, 1994; Liu et al., 1999). Both the mouse anti-human CD40 antibody mu5D12 and the humanized version of this antibody, ch5D12, displayed strong suppressive effects on the development of CNS white matter lesions and neurological deficit in the marmoset EAE model and showed no marked side-effects (Laman et al., 2002) (Boon et al., 2001). The same studies showed that intravenously injected anti-CD40 Mab into EAE affected common marmosets can gain access to the brain white matter lesions where CD40 molecules are prominently expressed on infiltrated macrophages and activated microglia (Laman et al., 1998b), as was found earlier in MS (Gerritse et al., 1996). This has raised the question whether ch5D12 also has a therapeutic effect on already existing lesions.

To address this issue we have monitored brain lesion development in 7 rhMOG-immunized monkeys by serial magnetic resonance imaging (MRI) at 2 weeks interval. Treatment with ch5D12 ($n=3$) or buffered saline (PBS) ($n=4$) was started once a lesion was detectable with MRI. At each time point the total volume and the mean T2 relaxation times of all brain white matter lesions were calculated, as a measure of demyelination and inflammation respectively. Moreover, the percentage increase of T1W signal intensity after intravenous injection of the contrast substance (GdDTPA) was determined as a measure of blood–brain barrier permeability ('t Hart et al., 2004d).

The results demonstrate suppression of lesion inflammation in all 3 ch5D12-treated monkeys while lesion enlargement was diminished in 2 of the 3 ch5D12-treated monkeys. These findings support the therapeutic potential of ch5D12 as a novel treatment for MS.

2. Material and methods

2.1. Animals

Seven common marmosets (*Callithrix jacchus*) were used for this study (see Table 1). The monkeys were housed in the animal facility of the Biomedical Primate Research Center in Rijswijk, The Netherlands. During the studies the marmosets were individually housed in spacious cages with padded shelter on the bottom. The monkey diet consisted of commercial food pellets for non-human primates (Special Diet Services, Whitham, Essex, England), supplemented with rice and fresh fruit. The monkeys had free access to drinking water. The experimental procedures of this study have been reviewed and approved by the institute's animal care and use committee.

2.2. EAE induction

EAE was induced by a single s.c. immunization with 100 μg rhMOG, a recombinant protein that represents the extracellular domain of human MOG, emulsified with complete Freund's adjuvant (Brok et al., 2000). The

Table 1
Individual characteristics of the monkeys

Code	Sex	Birth date (dd/mo/yr)	First lesion on MRI	1st clinical EAE (score 2)	Sacrifice (score≥3)
PBS treatment					
Monkey 1	M	24/08/98	day 90[a]	day 119 (29)	day 139 (49)
Monkey 2	M	16/09/98	day 53	day 63 (16)	day 67 (14)
Monkey 3	M	18/05/99	day 35	day 41 (6)	day 47 (12)
Monkey 4	M	18/12/98	day 35	day 41 (6)	day 52 (17)
Mean				14.3	23
ch5D12 treatment					
Monkey 5	M	24/08/98	day 69	day 73 (4)	day 94 (25)
Monkey 6	M	04/09/98	day 42	day 59 (17)	day 67 (25)
Monkey 7	M	12/09/98	day 33	none	day 52 without clinical EAE (22)

[a] Days after immunization.

expression of macroscopic signs of EAE was scored at least once daily by an independent observer using a previously described scoring system ('t Hart et al., 1998):

0	no clinical signs
0.5	apathy, loss of appetite and altered walking pattern without ataxia
1	lethargy and/or anorexia,
2	ataxia, sensory loss/blindness
2.5	hemi- or paraparesis
3	hemi- or paraplegia
4	quadriplegia
5	death attributable to EAE

Scores ≥ 2.0 represent different grades of neurological deficit, while 0.5 and 1.0 represent more general disease markers. Monkeys developing clinical score 3 or higher were sacrificed for ethical reasons by intravenous infusion of 400 mg sodium-pentobarbital (Euthesate; Apharmo, Duiven, The Netherlands).

2.3. Reactivity and dosing regimen of anti-CD40 antibody

We have used a chimeric antibody containing variable region elements from the mouse-anti-human CD40 antagonist antibody 5D12 and the human IgG4 constant region (Boon et al., 2002). When administered around the time of immunization ch5D12 was found to block clinical and neuropathological expression of EAE in common marmosets (Boon et al., 2001). The parent antibody mu5D12 was found to limit progression of EAE also when administered at a late stage of this disease (Laman et al., 2002). For the current experiment the first (loading) dose of ch5D12 (1 mg/ml/kg) was given by intravenous bolus injection. At day 1 and every second day thereafter ch5D12 (1 mg/ml/kg) was administered by intraperitoneal injection.

2.4. MRI measurements

High-resolution MRI experiments were performed using a 4.7 T horizontal bore NMR spectrometer (Varian, Palo Alto, CA), equipped with a high-performance gradient insert (12 cm inner diameter, maximum gradient strength 220 mT/m). A Helmholtz volume coil (∅ 85 mm) and an inductively coupled surface coil (∅ 35 mm) were used for radio frequency transmission and signal reception, respectively. Baseline measurements of each animal were collected before EAE induction. After EAE induction the animals were scanned once every 2 weeks until they reached disability score 3.0 (hemi-/paraplegia) at which stage they were sacrificed.

Anesthesia for MRI recording was induced by intramuscular injection of ketamine (1 mg in 100 μl per kg; ASP Pharma BV, Oudewater, The Netherlands) after which the animals were instrumented for mechanical ventilation by endotracheal intubation. The tail vein was cannulated for injection of gadopentetate dimeglumine (Gd-DTPA). The head was immobilized in a specially designed metal-free stereotactic device placed in an animal cradle, which was inserted into the NMR spectrometer. During the NMR-experiments the animals were ventilated with isoflurane (1%) in N_2O/O_2 (70/30). Expiratory CO_2 was monitored, and the body temperature was maintained at 37 °C with a heated water pad.

On a sagittal scout image, 35 contiguous coronal slices of 1 mm were defined covering the complete brain. The field of view was 4×4 cm^2, matrix: 128×128; zero-filled to: 256×256; in-plane resolution: 312.5 μm^2, two transitions. From these 35 slices the following MRI data sets were collected:

- Quantitative T2 maps, visualizing regions where tissue water is increased, e.g. by edema. These maps were obtained by a mono-exponential fit of five multi-echo images. TR = 5000 ms; TE = 17.5, 35, 52.5, 70 and 87.5 ms. The T2W images with a TE of 35 ms were used as for region of interest determination (ROIs, see also later).
- Quantitative GdDTPA enhanced T1W maps (GE-T1W), visualizing regions with increased vascular leakage. These maps were calculated from two T1W images (TR = 650 ms, TE = 11.5 ms) before and after a bolus of 0.3 mmol/kg GdDTPA (i.v., 12.5 min in circulation) with GE-T1W = $100*[(T1W_{post\ GdDTPA} - T1W_{pre\ GDTPA})/(T1_{pre\ GDTPA})]$. Pixel-intensities thus display the percentage increase in T1W signal intensity due to GdDTPA leakage.

Calculations of T2 and GE-T1W maps were done with a homemade software package developed in Interactive Data Language (IDL version 5.3, Research Systems, Boulder CO).

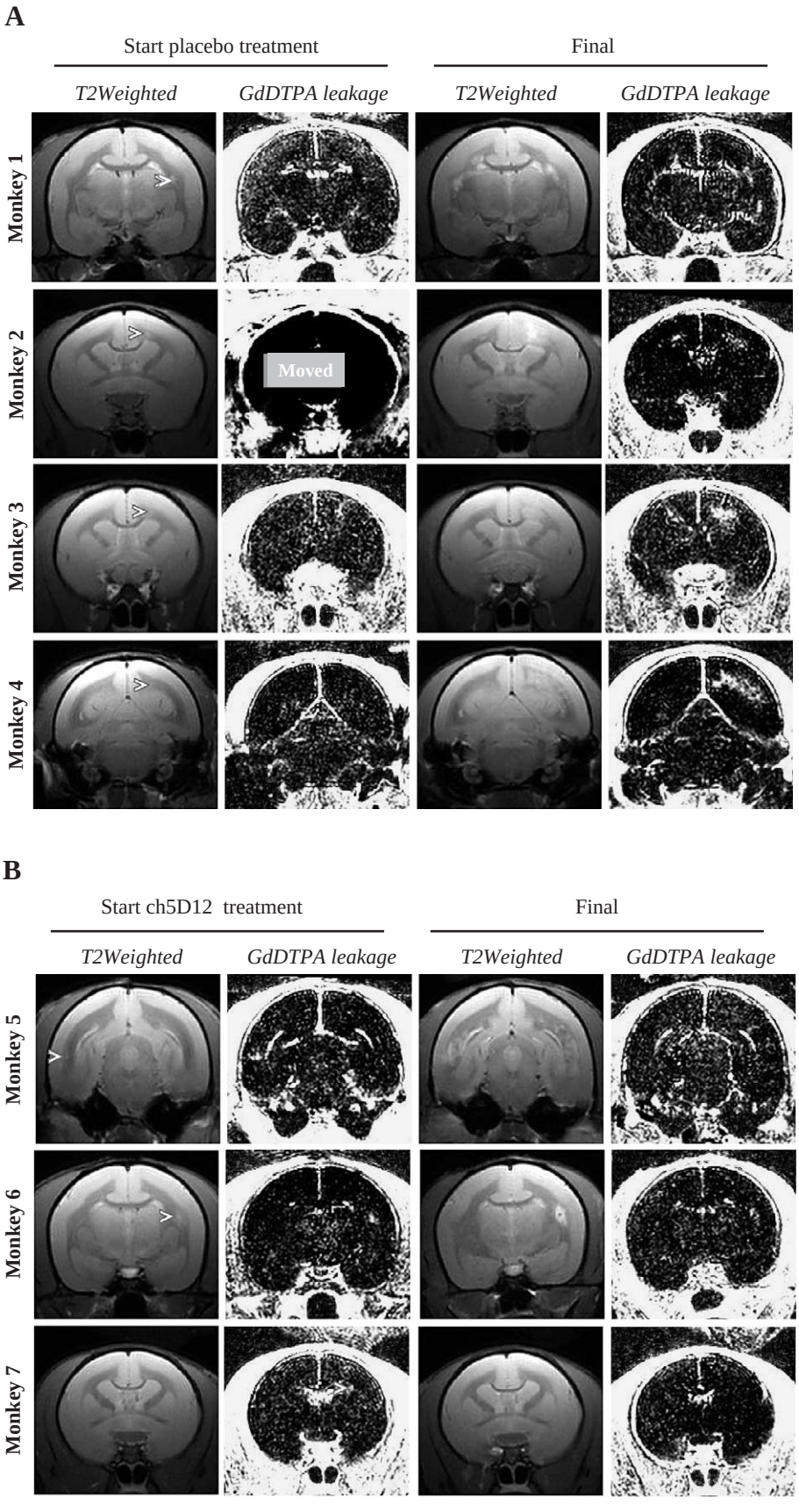
2.5. Histology

After formalin fixation, parts of the brain and spinal cord were embedded in paraffin and processed as described previously ('t Hart et al., 1998). In brief, the cerebrum and cerebellum were divided into 7 or 8 coronal cut parts and the spinal cord was dissected transversely. The extent of inflammation, demyelination and axonal pathology was evaluated on 3–5 μm tissue sections stained with hematoxylin and eosin (HE) to visualize infiltrated cells, Klüver Barrera luxol fast blue (LFB) combined with periodic acid schiff (PAS) for myelin and myelin degradation products, and with Bielschowsky silver impregnation for axons. The activity of the lesions was assessed as previously described ('t Hart et al., 1998). Immunostainings for detection of mononuclear cell subsets were performed with anti-CD20 for B-cells, anti-CD3 for T-cells and MRP-14 for macrophages.

3. Results

3.1. MRI characteristics of lesions developing in placebo-treated monkeys

The brain of rhMOG-immunized common marmosets contains two main lesion types. By far the most prevalent


A
Start placebo treatment
Final
T2Weighted
GdDTPA leakage
T2Weighted
GdDTPA leakage
Monkey 1
Monkey 2
Moved
Monkey 3
Monkey 4
B
Start ch5D12 treatment
Final
T2Weighted
GdDTPA leakage
T2Weighted
GdDTPA leakage
Monkey 5
Monkey 6
Monkey 7

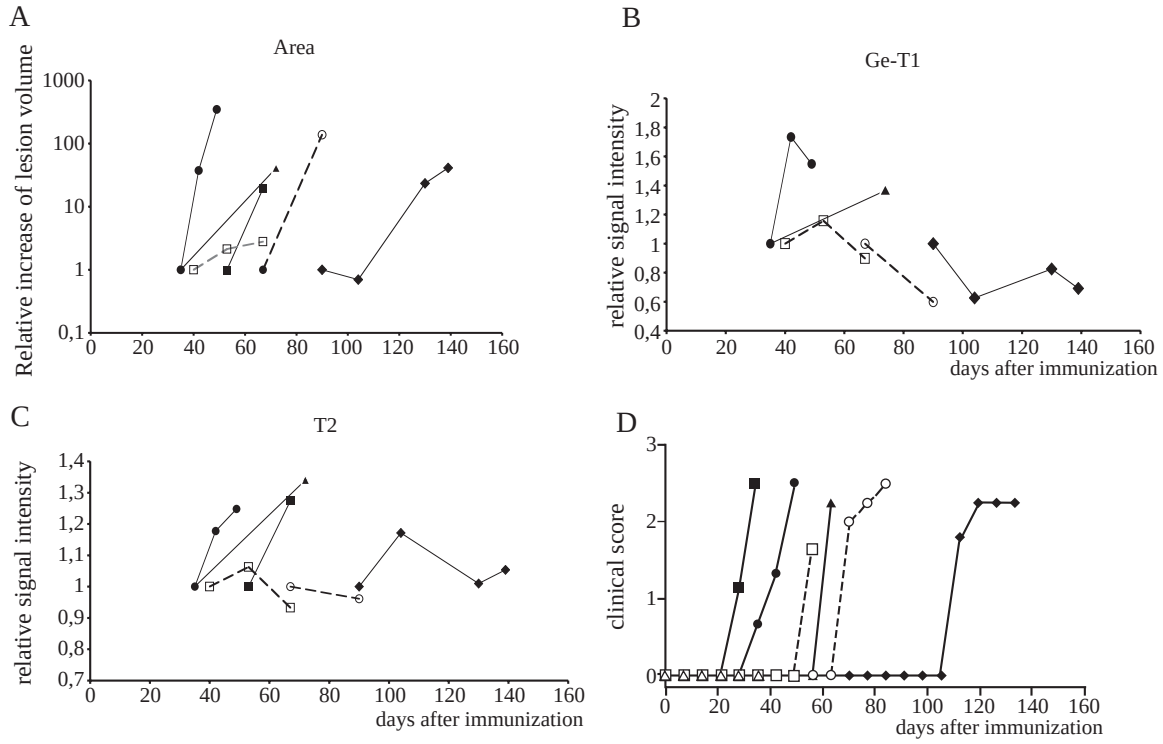


Fig. 2. Serial MR-imaging and clinical scores of rhMOG-immunized common marmosets: At about 2 weeks time interval MRI scans were made to assess the presence of lesions in CNS white matter. The recorded parameters were lesion volume (A), the relative signal intensity on T1-weighted contrast-enhanced images (B) and the relative increase of T2 signal intensity (C). The results were normalized by defining the values determined at the first appearance as 1. Monkeys placebo group: ▲ monkey 1; ■ monkey 2; ● monkey 3;◆ monkey 4. Monkeys anti-CD40 treatment group: ○ monkey 5; □ monkey 6; Δ monkey 7. Data of monkey 7 are lacking from figures A, B and C as the first lesion (see Fig. 1) appeared suppressed and no new brain lesions were formed. In B data from monkey 2 are lacking because the monkey moved during the scanning at the second time point. Daily clinical score of each monkey were recorded and averaged over a week. Graph D shows the week scores.

type resembles the early active pattern II lesions in MS, characterized by fulminant inflammation and demyelination, but without apparent axonal destruction. Demyelinated lesions with very limited inflammation, but significant axonal destruction are much rarer. The two lesion types can be distinguished with the quantitative imaging techniques that we have used in the current study ('t Hart et al., 2004d).

The first MRI-detectable sign of a lesion is increased permeability of the blood–brain barrier (BBB). This is visualized by extravasation of the intravenously injected contrast substance GdDTPA, giving rise to an increased T1 signal ('t Hart et al., 1998). The subsequent accumulation of edema can be visualized as a sharp increase of the T2 relaxation times. Hence, inflammatory active lesions appear as focal areas of increased signal intensity on T2-W images ('t Hart et al., 2004d).

The left part of Fig. 1A shows the brain slice in each of the 4 monkeys assigned to the sham group that contains the first MRI-detectable lesion (see arrow). From this time point onwards the monkeys were treated with PBS. The right part of Fig. 1A shows the markedly increased load of T2 lesions with clear GdDTPA leakage in the identical slice recorded just before the monkeys were sacrificed. Note that the duration of EAE differs substantially between individual monkeys (see Table 1), which likely reflects the outbred nature of the species (Brok et al., 2001).

Fig. 2 depicts the summarized MRI data of all 35 brain slices recorded at the consecutive time points. In all 4 sham-treated monkeys (solid lines) a sharp increase of the total volume (A) and the T2 relaxation times (B) of lesions was observed. This is typical for inflammatory active lesions, which is supported by the significant T1 signal enhancement by leakage of intravenous GdDTPA into the lesions (Fig. 2B). The late responder monkey 1 shows a somewhat deviant profile, namely a slower increase of the T2 lesion volume, a variable T2 intensity and a variable increase of the T1 signal intensity by GdDTPA leakage.

Fig. 1. Brain white matter abnormalities before start of treatment: The a priori condition set for this experiment was that treatment with ch5D12 Mab should be started as soon as a brain white matter lesion was detectable with MRI. Figure A shows the 4 monkeys that were assigned to the PBS-treatment group. Figure B shows the 3 monkeys that were assigned to the ch5D12 treatment group. The two left columns depict the situation at the start of treatment, while the two right columns depict the identical slice in the last MRI recording before the monkey was sacrificed. The arrows point at visible T2 lesions. The differential images visualizing brain barrier leakage were created by subtraction of T1-weighted images recorded before and after intravenous injection of 300 μM/kg Gd-DTPA. The data of GdDTPA leakage in monkey 2 at the first time point are lacking because the monkey moved during the scanning.

3.2. MRI characteristics of lesions developing in ch5D12-treated monkeys

The left part of Fig. 1B shows the brain slice in each of the 3 monkeys assigned to the ch5D12 group that contains the first inflammatory active MRI-detectable lesion (see arrow). From this time point onwards the monkeys were treated with anti-CD40 antibody. The right part of Fig. 1B shows the lesion load in the identical slice recorded just before the monkeys were sacrificed. The increment of the lesion load in this slice is substantially lower than in the sham-treated monkeys. Moreover, the much lower extent of GdDTPA leakage is indicative for a reduced activity of the lesions.

The summarized data of all 35 brain slices are given in Fig. 2 (dotted lines). As the brain of monkey 7 was completely devoid of lesions, this monkey is lacking in this figure. The figure shows a diminished enlargement of the T2 lesion load in monkey 6, but not in monkey 5 (Fig. 2A). However, the extravasation of GdDTPA and the increment of the T2 signal intensity observed in all sham-treated monkeys were absent in both monkeys, suggesting a complete suppression of inflammation within the lesions.

Despite this clear effect of the antibody on the activity of brain lesions in the model, we observed no delayed expression of clinical signs (Table 1; Fig. 2D).

3.3. Histology

To assess whether treatment with anti-CD40 affected inflammation and demyelination, extensive neuropathological analysis was performed. Fig. 3 shows histological

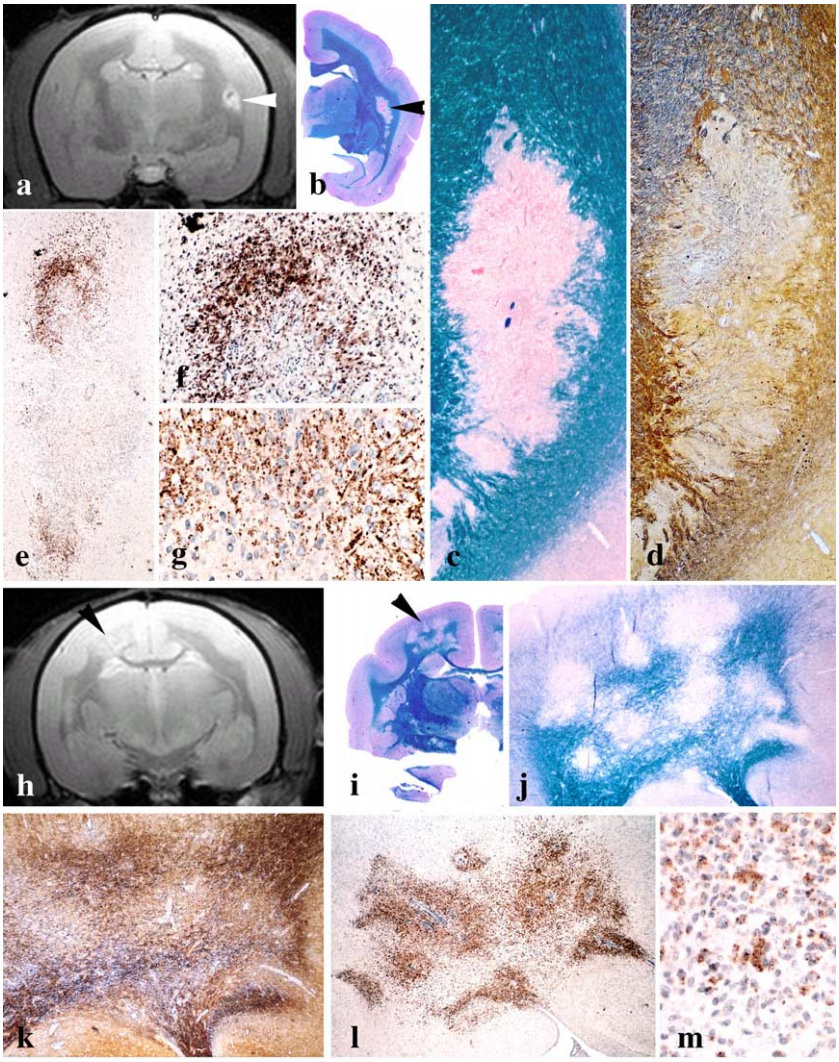


Fig. 3. Representative histology of brain white matter lesions: Representative examples of a rhMOG-immunized marmosets treated with ch5D12 (monkey 6; a to g) or placebo (monkey 2; h to m) with clinically active EAE. The pictures shown are a high contrast anatomical MR image (a, h, × 2) and a Klüver Barrera staining of the same slice showing myelin in blue (b and i, × 2). Demyelinated lesions are indicated by an arrowhead c) a magnification of the demyelinated lesion (c, × 32; j, × 15); a Bielschowski staining for axons (d, × 32 and k, × 15); e and l show MRP-14 staining of activated macrophages; f (× 70) shows the upper part of lesion depicted in e, indicating that demyelinating activity is only present at the border of this lesion; g (× 270, same area as f) and m (magnification of l; × 246) show MRP-14 positive macrophages with PLP-positive degradation products.

pictures of inflammatory active T2 lesions in the ch5D12-treated monkey 6 (a to g) and the placebo monkey 2 (h to m). The depicted hyperintense T2 region (h) in the placebo-treated monkey represents a clearly demyelinated lesion (i, j) with conservation of axons (k). The presence of many activated macrophages (l; MRP-14 positivity as an established activation marker (Brück et al., 1995; 't Hart et al., 1998) which contain phagocytosed myelin degradation products as visualized by staining for proteolipid protein (m), illustrates the active character of this lesion. The depicted GdDTPA-enhancing (not shown) and T2 hyperintense region (a) in the ch5D12-treated monkey also appears to represent a demyelinated lesion with limited axonal destruction. The staining for MRP-14 (e and f) and proteolipid protein (g) shows lower numbers of activated macrophages with myelin degradation products, illustrating the lower inflammatory activity in this lesion.

4. Discussion

CD40 is a surface expressed molecule on B cells and activated APC that delivers crucial co-stimulatory signals for the induction of autoreactive Th1-cells and autoantibodies in EAE (Brück et al., 1995; Ichikawa et al., 2002; Laman et al., 1996). Because of its central role in the induction of an adaptive immune response, as well as macrophage effector functions, CD40 is a potential target for intervention in the induction of autoreactive T-cells and antibodies in MS. Prevention of CD40 engagement with its ligand CD154 on activated T cells early in the disease process has a significant impact on the clinical and neuropathological expression of EAE in rodents (Gerritse et al., 1996; Girvin et al., 2002; Howard et al., 1999; Laman et al., 1998a; Samoilova et al., 1997) and non-human primate models (Boon et al., 2001; Laman et al., 2002). CD40 is prominently expressed within the CNS white matter lesions of MS patients as well as EAE-affected rodents (Gerritse et al., 1996) and non-human primates (Laman et al., 1998b). That CD40 bearing APC within the CNS, such as infiltrated macrophages as well as perivascular and parenchymal glia cells, contribute significantly to the pathogenesis of EAE has been elegantly shown in bone marrow chimeric mice (Becher et al., 2001).

In previous EAE intervention studies in human myelin immunized common marmosets, we have observed that therapeutic antibodies against human CD40 do not only gain access to CNS white matter lesions, but that this is associated with a reduced local expression of inflammatory mediators, such as MMP9 and TNF-α (Laman et al., 2002). Both factors are induced by CD40–CD154 interaction (Alderson et al., 1993; Miltenburg et al., 1995). The objective of the current study was therefore to investigate whether anti-CD40 antibody causes a reduction of inflammatory activity within existing CNS white matter lesions.

This was investigated in the rhMOG-induced EAE model that reproduces most critical immunopathogenic features of the myelin-immunized model (Brok et al., 2000; Genain et al., 1995, 1996, 1999; Raine et al., 1999). That the persistent activity and rapid increase in size of lesions can be recorded with MRI makes the MOG-induced EAE a highly useful preclinical model for therapy testing ('t Hart et al., 2004d). CD40 engagement was blocked with the chimeric anti-human CD40 Mab ch5D12, which in a previous study was shown to protect rhMOG-immunized marmosets against the clinical and neuropathological consequences of EAE (Boon et al., 2001). While in our previous study administration of the antibody was started at the time of EAE induction we chose to mimic in the current study more closely a future treatment situation in MS patients. Hence, treatment was started when active brain lesions could be detected with MRI. The read-out parameter for a treatment effect was brain MRI, in particular the T2 signal intensity, the T2W lesion volume and BBB leakage (Molyneux et al., 2001). The first and second MRI parameters mainly reflect inflammation as changes in the T2 signal of white matter are predominantly caused by altered distribution of tissue water, such as by vasogenic edema. We have included T2 lesion load quantification, as this parameter provides a useful marker of ongoing disease activity in MS (Molyneux et al., 1998).

Although the three ch5D12-treated monkeys displayed a somewhat different response to the treatment, the overall picture emerges that the increase of T2 signal intensity and T2W lesion volume was suppressed in the monkeys that received ch5D12 when compared to the sham-treated monkeys. However, despite the beneficial effects of ch5D12 on the MRI-detectable CNS pathology we observed no clear clinical effect of the antibody (Fig. 2D). The time intervals between the detection of the first lesion and progression to clinical scores 2 and 3 were in the same range in both treatment groups. The absence of a clear clinical effect was not entirely unexpected as there is a clear discrepancy between brain lesion activity and clinical scores in this model ('t Hart et al., 2004b). The partial effect of the antibody treatment on lesion enlargement and the absence of a clinical effect suggest that the main inhibitory activity of the anti-CD40 antibody is on the pathogenic mechanisms that cause inflammation and less on the pathogenic mechanisms that cause demyelination and axonal pathology. Confirmation that this is indeed the case awaits the implementation of imaging techniques for tissue destruction, such as magnetization transfer and diffusion weighted imaging or MR spectroscopy.

The exact working mechanism of the anti-CD40 therapy is unknown. Although the animals (both treated and controls) contained lesions with various activities (early active, late active, inactive), the numbers and localization of CD3 and CD20 in the lesions of ch5D12-treated animals did not seem to differ significantly from those in placebo treated animals. One of the critical factors in the EAE pathogenesis

that is produced by APC upon CD40 engagement is IL-12p40 (Ichikawa et al., 2002; Laman et al., 2002). The fact that the observed effects of ch5D12 on clinical and neuropathological aspects (Brok et al., 2002) and on MRI aspects of pre-existing lesions of myelin-induced common marmoset model of EAE can be reproduced with a neutralizing Mab directed to the IL-12p40 (Brok et al., 2002; 't Hart et al., submitted for publication) confirms the importance of the CD40-IL-12/IL-23 pathways as a target of therapy in MS.

The results of the current study in conjunction with two previous studies indicate that antibody blockade of CD40 is a potentially effective treatment of MS. Importantly, the ch5D12 Mab has no apparent side effects in the marmoset EAE model nor in other primate species (Boon et al., 2002). The beneficial clinical effect of anti-CD40 antibody was demonstrated in placebo-controlled experiments in two EAE models in marmosets, namely induced with human myelin (Laman et al., 2002) or rhMOG (Boon et al., 2001). In addition, the current study shows an inhibitory effect of anti-CD40 antibody treatment on already existing lesions. On the basis of these three studies we propose that clinical evaluation of the ch5D12 Mab in MS should be considered.

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