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—Review—

An Overview of Models, Methods, and Reagents Developed for Translational Autoimmunity Research in the Common Marmoset (*Callithrix jacchus*)

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Abstract: The common marmoset (*Callithrix jacchus*) is a small-bodied Neotropical primate and a useful preclinical animal model for translational research into autoimmune-mediated inflammatory diseases (AIMID), such as rheumatoid arthritis (RA) and multiple sclerosis (MS). The animal model for MS established in marmosets has proven their value for exploratory research into (etio) pathogenic mechanisms and for the evaluation of new therapies that cannot be tested in lower species because of their specificity for humans. Effective usage of the marmoset in preclinical immunological research has been hampered by the limited availability of blood for immunological studies and of reagents for profiling of cellular and humoral immune reactions. In this paper, we give a concise overview of the procedures and reagents that were developed over the years in our laboratory in marmoset models of the above-mentioned diseases.

Key words: experimental autoimmune encephalomyelitis, immunology, multiple sclerosis, nonhuman primate, rheumatoid arthritis

Introduction

Therapies based on biological molecules, monoclonal antibodies or (antagonists of) cytokines are becoming the standard of care in autoimmune-mediated inflammatory disorders (AIMID). The high species-specificity often precludes safety and efficacy testing in rodent models, thus creating a need for disease models more closely related to humans.

The common marmoset is a small-bodied Neotropical primate that provides useful models of human biology and disease [18]. In its natural habitat, being the north-eastern regions of Brazil, marmosets have an average lifespan of 10 years, but it can be 15 or even more than 20 years in captivity. In their natural habitat, both sexes are of similar size (approximately 20 cm, with a tail of 30 cm), although the males are slightly bigger. The body weight of adult monkeys ranges from 250 to 350 g; monkeys of 450 g body weight in captivity are obese. Sexual maturity is reached at 18 months. Mothers give birth to two twins or triplets per year. Most twins or triplets develop *in utero* as bone-marrow chimeras due to fusion of the placental bloodstream.

In response to the need for valid preclinical models

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Table 1. Advantages and disadvantages of the common marmoset in biomedical research

Advantage	
Proximity to humans	Genetics, (neuro) anatomy, immunology, physiology, microbiology.
Biology	Relatively small (300–350 grams) compared with other nonhuman primates (e.g., macaque species), high reproductive efficiency in captivity, lower caging and feeding costs compared with macaques, socially housed.
Conventional housing	Exposure of immune-shaping pathogens from the external milieu (e.g., gut microbiotica and environment) and from the internal milieu (e.g., opportunistic infection with herpes viruses such as the marmoset counterparts of Epstein-Barr virus and cytomegalovirus).
Outbred nature	Comparable genetic heterogeneity to the human population. Wild populations are not endangered.
Cross-reactivity	Biological therapeutics developed for human diseases e.g., monoclonal antibodies and cytokines, can be assessed for preclinical evaluation of efficacy, safety, and mechanism of action.
Bone-marrow chimerism	Twins or triplets are immunologically highly similar, and hence can be used in pairs for therapeutics studies. Twin siblings are mutually allotolerant, enabling adoptive transfer of cells between siblings.
Drug development	Cheaper due to small size, 10- to 20-fold less of an experimental drug is needed compared to macaques.
Disadvantage	
Costs	Relatively high compared with rodents or other non-rodent species.
Cross-reactivity	Limited availability of diagnostic reagents such as monoclonal antibodies for flow cytometry and immunohistochemistry.
Ethical	Are closer to humans compared with rodents, limited possibilities for experimental manipulations (e.g., transgenic experiments).
Size	Small size, difficult or impossible to perform certain procedures or techniques (e.g., MRI of spinal cord), small volume of blood or organs (e.g., lymph nodes) can be obtained to perform *ex vivo* experiments.

of AIMID, we have set up preclinical models of multiple sclerosis (MS), i.e., experimental autoimmune encephalomyelitis (EAE), and of rheumatoid arthritis, i.e., collagen-induced arthritis (CIA), in the marmoset [5, 7, 24]. These models are meant to replace, where possible and relevant, the often acute EAE and CIA disease models in larger primates, such as the rhesus and cynomolgus macaques [10, 25].

The common marmoset offers several practical advantages as a preclinical disease model (Table 1); namely, the small body size of the adult marmoset (250–350 g) requires much less test compound than needed for an adult macaque (4–10 kg). Also, the availability of immunologically comparable twin siblings provides an ideal setting for placebo-controlled efficacy evaluation of a new treatment, whereas their nonaggressive nature enables handling for routine procedures without the need of sedation.

Thanks to the pioneering work of Japanese researchers, new technologies, which have been so important for understanding mouse immunology, can now also be successfully applied to the marmoset. Examples are the creation of animals with transgene expression in the germ line [17], techniques related to embryonic stem cells [16], and the development of induced pluripotent stem cells [22].

On the other side, there are major challenges in immunology research in marmosets, including the high costs of the animals, ethical constraints and the genetic diversity. Hence, the maximum information should be collected from individuals. To achieve this, several technical hurdles have to be overcome. The small body size limits the collection of blood for immune profiling to ± 3.5 ml per month (=1% of the monkey's body weight). Another hurdle is the limited availability of validated reagents for immunophenotyping of leukocyte subsets by flow cytometry, for detection of antibody or cytokine levels in serum or cell culture supernatants with ELISA, and for immunohistochemical analysis of tissues.

The aim of this paper is to provide a comprehensive summary of the methodologies and reagents that were developed over the years in our mechanistic immunological research in the common marmoset.

Models of Autoimmune-mediated Inflammatory Diseases

Experimental autoimmune encephalomyelitis (EAE)

EAE is an autoimmune neuroinflammatory disease that shares clinical and pathological similarities with the

human neurological disease MS. In preclinical research, the mouse EAE model is not only used as a well-validated MS model, but also as a prototype autoimmune disease model in which principles of autoimmunity and tolerance have been developed. Analogously, the EAE model in marmosets can be used to study basic mechanisms of autoimmunity and tolerance in a more human-like immune system that is exposed to opportunistic and new infections.

EAE induction: EAE in marmosets is evoked by injection of myelin, myelin proteins, or myelin peptides formulated in adjuvant; small volumes of the emulsion (100 μl) are injected at four locations into the dorsal skin under alfaxalone anesthesia. Myelin oligodendrocyte glycoprotein (MOG) is the immunodominant myelin protein in marmosets [6]. For EAE induction, total human [19] or mouse myelin [6], recombinant MOG1-125 [2], or human MOG peptide 34–56 [8] can be used. EAE can also be induced with other myelin proteins, such as myelin basic protein (MBP) or proteolipid protein (PLP), but for these antigens, formulation with a strong bacterial adjuvant, i.e., mycobacterium (3 mg/ml) enriched with complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI, USA) and Bordetella pertussis, is mandatory [3].

Recombinant human MOG extracellular domain, comprising the sequence 1–125 (rhMOG), is produced in *Escherichia coli* and purified as previously described [11]. Synthetic MOG peptides are commercially purchased (Cambridge Research Biochemicals, Cleveland, UK). The inoculum for EAE induction contains 100 μg rhMOG or MOG34–56 in 200 μl phosphate buffered saline (PBS) and is emulsified in 200 μl CFA containing 1 mg/ml *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI, USA) by gentle stirring for at least 1 h at 4°C. Recently, we have shown that EAE can also be induced using rhMOG or MOG34–56 in incomplete Freund's adjuvant (IFA) [5] (manuscript in preparation). The absence of bacterial antigens in IFA creates a much more friendly animal model than induction with CFA, as the discomfort to the animals caused by the formation of ulcerative skin lesions at the immunization sites is significantly reduced.

Monitoring of the EAE course: The primary disease parameter is the clinical expression of EAE signs and symptoms. Marmosets developing EAE display different types of neurological deficits and lose body weight during periods of active disease. Clinical signs of EAE are scored by visual inspection once or twice per day by two independent observers. Overt signs of neurological deficit are recorded according to a documented semi-quantitative scoring system [20]. Briefly, 0=no clinical signs; 0.5=apathy, altered walking pattern without ataxia; 1=lethargy, tail paralysis, tremor; 2=ataxia, optic disease; 2.25=monoparesis; 2.5=paraparesis, sensory loss; and 3=para- or hemiplegia. Overt neurological deficit starts at score 2. For ethical reasons, monkeys are sacrificed once complete paralysis of limbs (score $\geq$3.0) is observed, or at the predetermined endpoint of the study.

The secondary disease parameter is the development of pathological abnormalities within the brain [21]. The disease course can be visualized by magnetic resonance imaging (MRI) using the same sequences as used for MS diagnosis and monitoring in a clinical setting. The sequences developed for the marmoset EAE model include qualitative ones for visualization of brain lesions and semiquantitative ones for the quantification of lesion activity. At the end point of an experiment, marmosets are sacrificed, and the brain and spinal column are removed. Usually the brain is cut into two symmetrical halves. One brain half is briefly fixed in 4% buffered formalin, and the other half is sectioned into smaller pieces, which are snap-frozen with liquid nitrogen in small aluminium containers. To assess the total load and the spatial distribution and size of lesions, a high contrast postmortem T2-weighted MRI scan of the fixed brain half is performed. With such scans, the size and spatial distribution of lesions can be visualized, guiding the dissection of the hemisphere for histological examination. After this, the tissue is processed for histological examination of inflammation, demyelination, and injury. The frozen brain half is used for immunohistochemistry analysis or DNA/RNA isolation for molecular analysis.

Tertiary and exploratory disease parameters include the analysis of humoral and cellular immune parameters (see below).

Collagen-induced arthritis (CIA)

CIA is an autoimmune inflammatory disorder primarily affecting the synovial joints. The model shares clinical and pathological similarities with rheumatic disease in humans and is accepted as a valid animal model for development of antirheumatic treatments. CIA in marmosets is gaining interest, not only as a valid RA model, but also as a potentially relevant model of frequently occurring comorbidities, such as dyslipidemia

and anemia [24].

CIA induction: Commercially available chicken collagen type II (chCII) (MD Biosciences, Zürich, Switzerland) is dissolved in 0.1 M acetic acid to a final concentration of 5 mg/ml at 4°C. This solution is mixed with an equal volume of CFA (Difco Laboratories). A stable emulsion is prepared by gentle stirring of the protein/CFA emulsion for 60 min at 4°C. CIA is elicited by injection of 0.4 ml emulsion into the dorsal skin distributed over 4 spots of 100 μl. The amount of CII injected into each monkey is thus 1 mg/animal. When overt clinical signs of arthritis do not develop within 28 days post sensitization (psd), a booster immunization is given with CII in IFA subcutaneously at the flank.

Monitoring the disease course: The primary CIA parameter is the presence of overt clinical signs of CIA, which are scored once or twice per day by two independent observers using a previously described semiquantitative scale [25]. Briefly, 0=no clinical symptoms; 0.5=fever (>0.5°C); 1=apathy, loss of appetite, and weight loss; 2=warm and tender joints without soft tissue swelling (STS); 3=moderate STS, but normal flexibility of affected joints; 4=severe STS with joint stiffness; and 5=severe disease necessitating humane killing. Once per week, monkeys are sedated for a more detailed diagnosis. At that time, individual joints are inspected. The presence of joint inflammation is recorded on a semiquantitative scale (0=no arthritis, +/−=minimal, +=moderate, ++=severe).

The secondary disease parameters are serum biomarkers of inflammation (serum amyloid A) and bone remodelling (alkaline phosphatase), and urine biomarkers of joint erosion [hydroxylysyl pyrridinoline (HP) and lysyl pyrridinoline (LP)]. Urinary excretion of the collagen cross links HP and LP are determined twice weekly, starting from the day of CIA induction. For that purpose, each animal's urine is collected overnight in a metabolic cage (24 h). After centrifugation, the clear supernatant is isolated and stored at −20°C. Reverse-phase HPLC is used to determine HP and LP levels in hydrolyzed urine samples as described previously [23]. The levels of HP and LP are normalized to urine creatinine levels (nmol levels per mmol creatinine) to compensate for possible dilution by spilled drinking water.

The tertiary disease parameter is the histological scoring of joint inflammation and erosion according to Pettit *et al.* [14]. Just like in the EAE model, immune profiling of the monkeys provides exploratory disease parameters.

Immunological Techniques

Hematology and serology

Similar to clinical studies, hematology and serology analyses are performed to detect possible side effects of a treatment. Both analyses are performed using fully automated analyzers, i.e., a Sysmex XT-2000i hematology analyzer (GMI, Ramsey, MN, USA) and COBAS Integra 400 Plus serology analyzer (Roche, Indianapolis, IN, USA), respectively. The normal values for a range of hematology parameters are listed in Table 2 and for serology parameters in Table 3.

Humoral autoimmune profiling

The pathogenesis of autoimmune diseases involves the synergy of humoral and cellular adaptive immune factors. Although the assays used in marmosets are essentially the same as used in humans, modifications are made to accommodate for small sample volumes and the need for longitudinal monitoring, with the aim to maximize the information that can be obtained from individual animals.

In brief, 96-wells ELISA plates (Greiner Bio-One BV, Alphen ald Rijn, Netherlands) are coated with 0.5 μg/well antigen of interest, such as MOG for the EAE model, or collagen for the CIA model. Bound IgM Ab is detected using alkaline phosphatase-conjugated goat-anti-monkey IgM (Rockland Immunochemicals, Gilbertsville, PA, USA) and bound IgG Ab is detected using polyclonal alkaline phosphatase-conjugated rabbit-anti-human IgG (Abcam, Cambridge, UK). As marmoset IgG subclasses have not been identified to date, ELISA for the detection of the IgG subclasses has not been set up yet.

For normalization, results of the Ab assays are expressed relative to a reference curve made from pooled necropsy plasma present on each plate. Titer calculations are made with the help of homemade software, namely Auditable Data Analysis and Management System for Elisa (ADAMSEL, developed by Dr. E. Remarque, BPRC, The Netherlands) (www.malariaresearch.eu).

Isolation of mononuclear cells (MNCs)

Whereas in human immunology MNCs from venous blood are normally used for immunological experiments, MNCs from the spleen and (more rarely) lymph nodes are used in mice and rats. In marmosets, MNCs from blood as well as lymphoid organs can be used, enabling

Table 2. Hematological reference values of male (n=114) and female (n=52) marmoset monkeys older than 2 years

Cell type	Values		Unit
	Male	Female	
Leukocyte count	1.24–9.76	0.43–11.14	10^9/l
Erythrocyte count	5.11–8.00	4.61–807	10^{12}/l
Hemoglobulin	7.6–11.00	7.0–11.1	mmol/l
Hematocrit	0.4–0.57	0.36–0.57	l/l
Mean cell volume	65.5–827	65–82.0	fL*
Mean cell hemoglobin	1,267–1,588	1,261–1,597	amol**
Mean cell hemoglobin concentration	18.4–20.2	18.3–20.6	mmol/l
Platelets	210–931	139–1003	10^9/l
Red cell distribution width	26.6–45.3	25.6–44	fL
Red cell distribution width-cell volume	11.0–19.2	10.1–19.5	%
Platelet distribution width	8.1–14.2	7.6–14.1	fL
Mean platelet volume	83–11.7	8.0–11.7	fL
Platelets large ratio	10.8–39.3	9.1–39.0	%
Neutrophils	0.0–5.93	0.00–7.13	10^9/l
Neutrophils	20.00–82.74	25.48–77.96	%
Lymphocytes	0.4–4.3	0.6–4.0	10^9/l
Lymphocytes	14.76–76.74	16.35–74.32	%
Monocytes	0.0–0.5	0.0–0.7	10^9/l
Monocytes	0.0–6.94	0.0–8.39	%
Eosinophils	0.0–0.1	0.0–0.1	10^9/l
Eosinophils	0.0–1.21	00–2.51	%
Basophils	0.0–0.1	0.0–0.1	10^9/l
Basophils	0.0–1.07	0.0–1.61	%

*fL = 10^{-15} liters, **amol = 10^{-18} moles.

Table 3. Normal values of chemical compounds in blood in male (n=114) and female (n=52) marmoset monkeys older than 2 years

Compound	Values		Unit
	Male	Female	
Albumin	27.81–55.01	29.08–53.86	g/l
Phosphate Alkaline	0.0–393.7	0.0–439.8	U/l
ALAT	0.0–164.4	0.0–497.2	U/l
ASAT	0.0–500.5	0.0–475.2	U/l
Bilirubin total	0.6–4.0	0.7–3.8	umol/l
Calcium	1.85–2.85	1.8–2.85	mmol/l
Cholesterol	1.96–6.07	1.43–4.97	mmol/l
Chloride	100.3–113.4	97.8–116.7	mmol/l
Carbon dioxide	11.0–32.3	10.7–30.1	mmol/l
Creatinine	33–67	33–67	umol/l
Gamma glutamyltransferase	0.0–15.2	0.0–8.7	U/l
Glucose	1.18–13.38	1.49–11.87	mmol/l
Iron	5.82–40.28	3.09–50.58	umol/l
Potassium	0.68–5.11	1.8–3.73	mmol/l
Lactate dehydrogenase	0.0–1291	0–1,997	U/l
Sodium	143.1–154.8	141.1–154.98	mmol/l
Phosphatase	0.34–1.77	0.44–1.79	mmol/l
Total protein	45.2–76.6	45.2–76.3	g/l
Urea	3.3–15.56	3.17–12.78	mmol/l

the parallel profiling of both compartments. The observation of a variable redistribution of activated lymphocytes over both compartments during the course of EAE underlines the relevance of this multi-compartment analysis in translational immunology.

Blood volumes of 1–1.5 ml are sufficient for the performance of routine cellular immune assays, such as lymphocyte phenotyping by flow cytometry and T-cell

proliferation as a read-out of antigen reactivity. Such blood volumes are drawn from the femoral vein and collected into EDTA or heparinized vacutainers (Greiner, Sölingen, Germany). After centrifugation for 10 min at 931 × *g*, plasma is collected and stored frozen at −20°C or −80°C. The cell pellet is resuspended to twice the original blood volume in PBS or RPMI medium (RPMI supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Bleiswijk, Netherlands)). The preferred method for isolation of marmoset peripheral blood mononuclear cells (PBMCs) is to layer diluted venous blood onto lymphocyte separation medium (LSM®, ICN Biomedicals Inc., Aurora, OH, USA) in Leucosep tubes (Greiner Bio-One BV) followed by centrifugation at 1,341 × *g*. Other methods, such as Ficoll, have proven to be suboptimal for the isolation of PBMCs from marmosets.

For isolation of the granulocyte fraction from venous blood, red blood cells are lysed for 5 min at RT in lysis buffer (containing NH_4Cl, $KHCO_3$, EDTA, pH 7.3). After separation in Leucosep tubes, PBMCs can be collected from above the filter, whereas the granulocytes can be collected from the bottom of the tube. The standard methods used for the isolation of human granulocytes, such as dextran sedimentation or percoll gradient centrifugation, do not work for marmoset cells.

Frequent sampling of small blood volumes (<100 μl), such as for pharmacokinetic/ pharmacodynamic analysis, can be done via a needle prick method into the saphenous vein of a conscious monkey. Blood is collected with a pipet and immediately diluted with 9 volumes buffered saline in EDTA- or heparin-coated tubes (Greiner Bio-One BV). After centrifugation (5 min, 400 × *g*), blood cells are pelleted, and the 10-fold diluted plasma is stored at −20°C or −80°C until analysis. The cell pellet can be resuspended in the desired medium and further processed for small-scale cellular immune assays, such as flow cytometry or DNA/RNA isolation.

For the isolation of MNCs from the spleen or lymph nodes, organs are squashed through a 70 μm cell strainer (BD Bioscience, Bedford, MA, USA). Splenocytes are then layered on top of LSM followed by centrifugation at 930 × *g* without brakes. MNCs from lymph nodes can be isolated without LSM by centrifugation at 673 × *g*.

Specific lymphocyte subsets are isolated from blood or, when substantial cell numbers are needed from the spleen, by using magnetic beads coated with monoclonal antibodies against, for example CD4, CD20 or CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany).

Assaying antigen reactivity of MNCs by proliferation

Similar to human and mouse immunology, the most frequently used assay to probe the reactivity of MNCs with antigens is quantification of DNA synthesis during proliferation via the incorporation of ^{3}[H]-thymidine. To this end, MNCs are cultured in triplicate at a cell density of 2×10^5 per well in 96-well U-bottom plates and stimulated with selected antigenic proteins and/or peptides, i.e., rhMOG and MOG peptides, in EAE studies and CII or CII peptides in CIA studies. Control cultures are not stimulated. Ovalbumin is used as a negative control antigen, and the mitogen ConA is used as a positive control. All antigens are tested at 5 μg/ml. After 48 h of culture, 50 μl supernatant is harvested from each well for cytokine production analysis with ELISA (see below) after which 0.5 μCi/well of ^{3}[H]-thymidine is added to the cells. Incorporation of radiolabel is determined after 18 h using a matrix 9600 β-counter (Packard 9600; Packard Instrument Co., Meriden, CT, USA). Results are expressed as the stimulation index, that is, the ratio of radiolabel incorporation in stimulated versus control wells. Stimulation index values ≥2 are considered to be positive.

The carboxyfluorescein succinimidyl ester (CFSE) vital dye dilution assay is used to phenotype the MNCs that proliferate against an antigen. In brief, 10^6 MNCs from blood, the spleen, or lymph nodes are incubated with 1.5 μM CFSE (Fluka, Deisenhofen, Germany) for 7 min at RT. After extensive washing, the cells are cultured similar to the ^{3}[H]-thymidine incorporation assay. Instead of 3 days, the cells are cultured for 7 days with stimuli of choice, after which the cells are stained with monoclonal antibodies (mAb) against human CD markers and analyzed by flow cytometry.

MNC phenotyping

Typically, 100,000–200,000 cells are used per staining, which is performed in a 96-well V-bottom plate (Greiner Bio-One BV). Most commercially available mAbs for flow cytometry have been raised against rodents, humans, or rhesus macaques. To analyze marmoset cells, a large panel of antibodies against human cells was tested for cross-reactivity [1]. This yielded a useful set of antibodies, of which the most commonly used are listed in Table 4.

Table 4. Most commonly used antibodies for flow cytometry

Antibody	Clone	Provider*
CD3	SP34-2	1
CD4	MT310	2
CD8	LT8	3
CD16	3G8	1
CD20	H299	4
CD27	M-T721	1
CD40	B-B20	5
CD45RO	UCHL1	4
CD56	Ncam16-2	1

1. BD Biosciences; 2. Dako; 3. Serotec; 4. Beckman Coulter; 5. Millipore.

Table 5. ELISA kits for marmoset culture supernatant

Cytokine	Provider*
IL-2	1
IL-10	1
IL-13	1
IL-12/23p40	1
IL-17A	1, 2
IFN-γ	1
TNF-α	1

*1, U-CyTech Biosciences; 2, eBioscience.

Table 6. Primer and probe combinations used to determine cytokine and surface marker expression by qPCR

Marker	Forward primer 5'-3'	Reverse primer 5'-3'	Probe*
IL-1β	TGGTCCTAAACAGATGAAGTGC	GTAGTGCTGGCGGGAGAGT	85
IL-2	AAGTTTTACATGCCCAAGAAGG	AAGTGAAAGTTTTTGCTTTGAGCTA	65
IL-4	CTAAAACGGCTGGACAGGAA	CCTTCACAGGACAGGAGTTCA	64
IL-6	CCAATCTGGATTCAATGAGGA	AACTCCAAAAGACCAGTGGTGA	40
IL-7	TGCACCAGCAAGGTTAAAGA	CCAAACTCTTTGTCGGTTGG	37
IL-10	GTTGCCTTCAGCAGAGTGAA	GCAACCCAGGTAACCCTTAAA	67
IL-17A	CCTCATTGGTGTCACTGCTG	TGCAATTCCTGCCTTCACTA	146
IL-17F	CAGCGTGTTCCCTTGTCAC	CCCAGGTGACGGTGTAATTC	10
IFN-γ	GGAGAGAGGAGGGTGACAGA	TTGGATGCTCTGGTTGTCTTTA	21
TNF-α	GGACGAGCTCTCCAAGGACT	GTCACTCGGGATTCGAGAAG	81
BLyS	CCTATTCCATGGGACATCTGA	CATCGAAACAAAGTCACCAGAC	6
APRIL	CCTGAATGACTACCGAGAGAGTG	TCGGTCATAGCCATCCAAGT	29
TACI	GAAGCAAGTCCAGCTCTCCA	AGCCCCAGTGTGCTGTAGAC	29
CD3	AGGCAAGAGTGTGTGAGAACTG	GATGCAGATGTCCACTATGACAA	10
CD4	TCTGTGAAGTGGAGAGCAAAAA	TGACAGTCAATCCGAACACC	10
CD8	TCATGTACTTCAGCAGCTTCG	GCTCTGGTGTGGGTGGTG	4
CD19	CAGCCCCGTCTTATAGAAACC	CACTGTCCGGCTCCTCATAG	35
CD28	TGGCCCTTAGTGTTTTCTGG	GTCATGTTCATGTAATCACTGTGC	68
Granzyme B	ACAGTACAGTTGAGTTGTGTGTGG	GAGTCCCCCTTAAAGGAAGC	64
Perforin	CCACGTGAAATTCCGCTATC	AGACTCTGGCGGGCATATT	81

*Probe numbers correspond to the Universal ProbeLibrary of Roche, Indianapolis, IN, USA.

Assaying cytokine production with ELISA

Production of cytokines can be quantified at the protein level by ELISA and at the mRNA level by qPCR. Protein levels are routinely measured in 48 h culture supernatants. Table 5 shows the ELISA kits that can be used to detect marmoset cytokines.

Immune profiling by quantitative polymerase chain reaction (qPCR)

With the marmoset genome published online, it is now quite easy to design primer sets for detecting mRNA transcript levels of biologically relevant molecules in cells or tissues with qPCR. RNA is isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Random hexamer primers are used for cDNA synthesis. Expression levels of specific mRNAs are determined by qPCR using iTaq Supermix and a CFX96 Real-Time PCR Detection System (both from Bio-Rad, Hercules, CA, USA). Primers and probes used are listed in Table 6. Probe sequences were obtained from the Universal ProbeLibrary set for human (Roche, Indianapolis, In, USA). Primer sequences were developed using the Universal ProbeLibrary Assay Design Center from Roche. As the marmoset is not present in the standard list of organisms in the Assay Design Center from Roche, a few extra steps need to be taken compared with working with human or rodent samples. The gene of interest is

searched in Ensembl, and the cDNA sequence is copied into the "sequence" field in Assay Design Center after selecting "Other Organisms". A manual assessment of the primer/probe combinations for intron spanning is required to prevent amplification of genomic DNA. The primer-probe combinations are validated by making of a serial dilution of the cDNA. Transcript levels are routinely normalized against the reference gene Abelson (ABL).

Assaying cytotoxic T cells

^{51}Chromium-labeled Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) are used as target cells to test the cytotoxic potential of T cells. B-LCLs are generated by infection of PBMCs with EBV strain B95-8 (see below). B-LCLs are incubated for 1 h at 37°C with ^{51}chromium, pulsed subsequently with antigenic peptide or protein and then thoroughly washed with PBS. Pulsed B-LCLs are mixed with effector T cells at 1:1, 1:4, and 1:16 ratios in 96-well U-bottom plates and incubated for 5 h at 37°C in culture medium. Thereafter, 100 μl supernatant is collected to measure the amount of radiolabel released from lysed cells in a γ-counter. Negative controls in this assay are peptide-pulsed target cells without T cells (spontaneous release) or peptide-pulsed target cells lysed with 1% Triton X-100 (maximum release). Results can be expressed as percentage killing, being (T-cell induced − spontaneous)/(maximal − spontaneous) × 100%.

Generation of Primary and Virus-Transformed Cell Lines

Generation of cell lines

For immunological research, primary T-cell lines as well as Herpesvirus-transformed T-cell and B-cell lines can be used. Transformed B-cell lines are a convenient source of autologous antigen-presenting cells for $CD4^+$ helper and $CD8^+$ cytotoxic T cells or for molecular studies, such as peptide binding assays [4]. Transformation of T cells, such as with *Herpesvirus saimiri* can change the function of T cells, but usually preserves the specificity [13]. Transformation of T-cell lines can be of use when a large number of T cells of certain specificity is needed, such as for the evaluation of a broad antigen panel.

Primary T-cell lines

Mononuclear cells isolated from secondary lymphoid organs of immunized animals are seeded into 24-well plates (Greiner, Sölingen, Germany) at a cell density of 10^6/well and stimulated with 10 μg/ml rhMOG or MOG peptide. Every 2 or 3 days, half of the culture supernatant is replaced with fresh medium containing 20 U/ml recombinant human IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, USA); cultures are split when needed. After 14 to 21 days of culture, cells are tested for specificity via ^{3}[H]-thymidine incorporation assay and phenotyped by flow cytometry. Antigen-specific T-cell lines can be used for functional assays. Cultures not yet specific for a certain antigen are restimulated with irradiated EBV-transformed B cells added at a 1:1 ratio to the T cells and 10 μg/ml antigen. Marmoset T cells can maximally be restimulated 3 to 5 times.

B-cell transformation

B cells are generated by infection of PBMCs with EBV strain 95-8. The B95-8 strain is derived from a human EBV isolate from infectious mononucleosis that has been kept for several decades in cottontop tamarin cells [15]. Briefly, lymphocytes are isolated from heparin/EDTA blood according to the LSM method described above, and are incubated with EBV supernatant for 1.5 h at 37°C. After incubation, cells are diluted with culture media containing 1 μg/ml PHA at a 1:1 ratio, and are plated in a 24-well plate, 2 ml/well. After 5 days, half of the culture media is replaced with fresh media (without PHA). It may take 2–6 weeks for B-cell lumps to form, which can be moved to a T25 culture flask. In the meanwhile, half of the media is replaced every 4 days.

Pathology

Magnetic resonance imaging (MRI)

The marmoset is a very useful model of neuroinflammatory and neurodegenerative diseases. The possibility to examine brain pathologies with clinically relevant magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) techniques is an enormous advantage. Although abnormalities are also developing in the spinal cord, MR imaging of the spinal cord in a living animal is technically highly challenging, due to the very small diameter and movement artefacts caused by breathing.

We have used MRI techniques *in vivo* for the longitu-

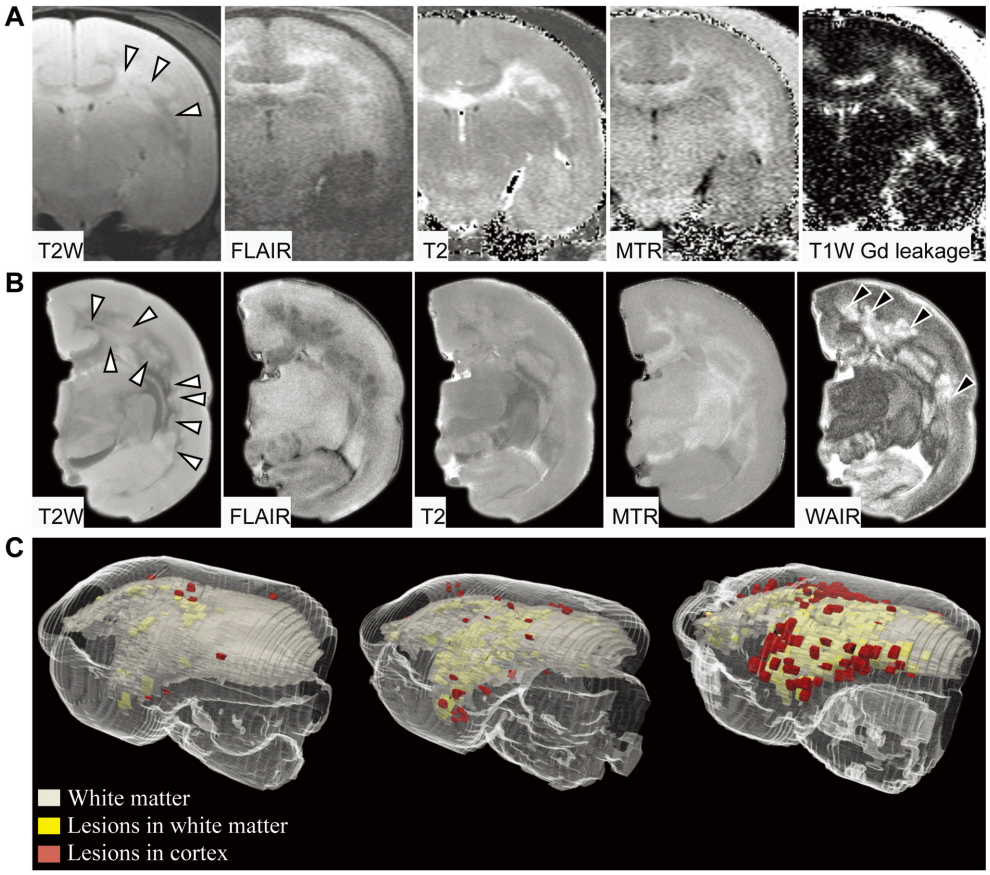

Fig. 1. Magnetic resonance imaging (MRI) sequences of marmoset EAE brain. A. MRI images were collected *in vivo* for animal M06061 at the time of clinically evident EAE symptoms. In each figure, the same slice position is shown. See also Table 7 for an explanation of the several MRI techniques. Arrowheads in the T2W image point to white matter lesions. Unique tor *in vivo* MRI is the possibility to measure the integrity of the blood brain barrier with intravenously injected gadolinium-based contrast agent. The depicted T1W Gd leakage image displays the increase in MRI signal intensity as a result of contrast leakage. B. The brain of the same monkey (M06061) with EAE symptoms was used for a postmortem MRI analysis. As with postmortem material, a higher signal intensity results in an increase in lesion detail. Arrowheads in the T2W image point to lesions in the white matter. Suppression of the signal intensity of the white matter, i.e., WAIR imaging, improves the detection of cortical grey matter pathology. Lesions in the cortex are indicated by arrow heads in the WAIR image. C. Three animals with increasing levels of MRI abnormalities in the brain are shown with almost identical EAE scores (left brain=M07076 EAE score 2.25; middle brain=Mi12699 EAE score 2.25; right brain=M07079 EAE score 2.5). 3D data sets are generated from postmortem MR images. Lesions in the white matter were determined on T2W images, and lesions in the cortex were determined on WAIR images.

dinal analysis of brain pathology in live animals and post mortem to determine the size and spatial distribution of pathological abnormalities at high contrast (Fig. 1). In T2-weighted (T2W) MRI, one of the intrinsic MR properties of protons, i.e., the T2 relaxation time, of tissue water is used to create contrast. As all pathological changes in a tissue are accompanied by changes in water content, T2W MRI is the most sensitive and thus frequently used diagnostic parameter. Other MRI parameters that have been developed and validated for the marmoset brain white matter (WM) are MR contrast-enhanced T1-weighted (T1W) images, and magnetic transfer ratio (MTR) images (Table 7). For quantification of pathological changes, T1 and T2 relaxation time images have been developed. Inversion recovery based images, in which WM and GM signals are suppressed, can be made for detection of grey matter (GM) pathology, which is found frequently in the marmoset EAE

Table 7. MRI techniques used for visualization of abnormalities in the brain of marmosets

Parameter	Characteristic
T1-weighted	Detects disappearance of tissue, e.g., due to demyelination or axonal injury. T1W images are sensitized for the T1 relaxation time (spin-lattice relaxation), a time constant (~ seconds) that describes the rate in which the longitudinal component of the magnetization vector recovers.
T1-weighted + contrast agent (Gadolinium based)	Visualization of the permeability of the BBB. Data are shown as the percentage increase in signal intensity due to presence of the leaking MR contrast agent in the brain parenchyma.
T2-weighted	Used to determine lesion volume (voxel or mm^3). T2W images are sensitized for the T2 relaxation time values.
T2 relaxation time images	T2 values are sensitive to changes in water content (edema) and demyelination. T2 images display T2 relaxation time values (spin-spin relaxation), a time constant (~ tens of milliseconds) describing the decay of the magnetization vector in the transversal plane.
Magnetization transfer ratio	The MTR is reduced by demyelination and edema. Measures the ratio of protons of macromolecule-bound water and freely moving water. The macromolecule-bound protons of water are saturated by a magnetization transfer pulse, and this saturation is transferred to the protons of freely moving water. Data are shown as the percentage decrease in signal intensity due to the saturation pulse.
Inversion recovery image	Images in which the signal arising from a specific fluid or tissue, such as CSF, white matter, or grey matter, is suppressed. Suppression of the white matter signal facilitates the detection of grey matter lesions.
Diffusion tensor imaging	Diffusion MRI measures the diffusion of freely moving water molecules in tissue. The presence of cell boundaries may restrict this diffusion. In diffusion tensor imaging (DTI), the preferred direction of the water diffusion is calculated. For an intact axon, this will be mainly along the direction of the myelinated axons, as the compact myelin sheaths will limit the diffusion of water perpendicular to this direction. In an injured axon, water will also diffuse perpendicular to the direction of the axon.

model.

MR imaging of fixed brain before sectioning is a powerful technique for defining the spatial distribution and size of abnormalities and their initial characterization. To reduce unwanted MR signal-destroying susceptibility artefacts, which arise on the tissue/air boundary, the formalin-fixed brains are submerged in a perfluoropolyether (Fomblin, Ausimont, NJ, USA). This procedure has been successfully used for the precise characterization of pathological changes in the brain WM and GM of EAE-affected marmosets.

Histology and immunohistochemistry

Histology is performed on formalin-fixed paraffin-embedded tissue. During EAE research, brain, spinal cord, and optic nerve tissues are used (Fig. 2). Brain samples, including the cerebrum and cerebellum, are divided into 7 or 8 coronal sections, and spinal cord and optic nerves are sectioned transversely into 10 to 15 pieces. Subsequently, all sections are embedded in three or four paraffin blocks. The extent of inflammation, demyelination, and axonal abnormalities is assessed on 3- to 5-μm-thick slices stained with 1. hematoxylin/eosin to visualize infiltrating cells, 2. Luxol fast blue combined with periodic acid-Schiff stain to show myelin and myelin-degraded products, and 3. Bielschowsky's silver stain for impregnation of axons.

For CIA research, at necropsy, hands and feet are removed and fixed in 4% phosphate-buffered formalin. After fixation, one finger of each extremity is excised for histopathological analysis and decalcified for at least 3 weeks in Kristensen's solution (17% formic acid in 1M NaOH, pH 2.2). Decalcified bones are washed in tap water for 16 h, dehydrated in ethanol/toluene, and embedded in paraffin. Sections of 2-μm-thick are cut and stained with hematoxylin and eosin.

Immunohistochemistry can be performed on frozen and formalin-fixed paraffin-embedded tissue. The monoclonal or polyclonal antibodies used for the marmoset were raised against human targets and preselected for cross-reaction with the marmoset [9]. A selected antibody was first tested at two concentrations on acetone-fixed snap-frozen tissue. When no staining was observed, the antibody was tested on paraformaldehyde-fixed frozen tissue. In the case of weak staining, a Tyramide Signal Amplification Kit (TSA, Invitrogen) can be used to amplify the signal. Also, as the background staining increases, inclusion of good controls in the analysis is essential.

Immunohistochemistry procedures in the marmoset

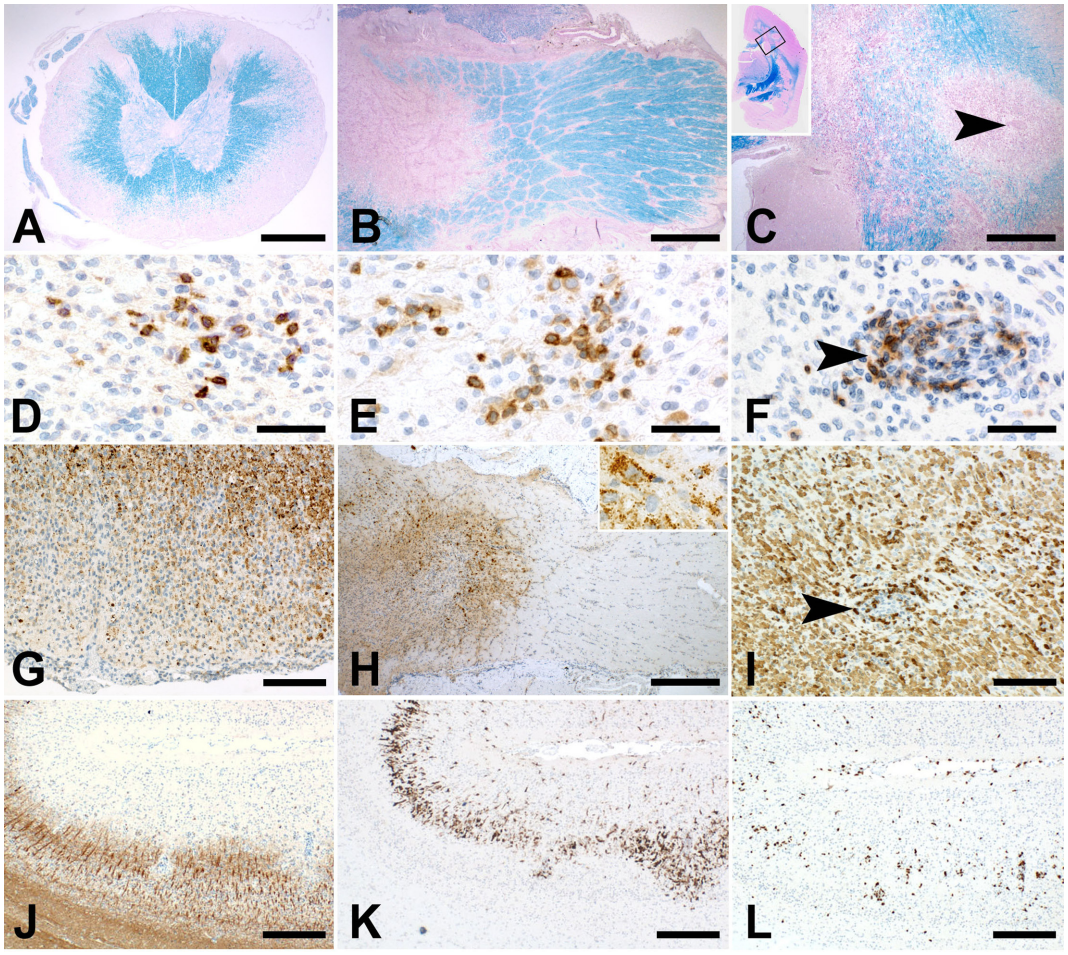

Fig. 2. Histology and immunohistochemistry of the spinal cord, optic nerve, and brain of monkey M06061, which was sacrificed with EAE symptoms. The MRI data from this monkey are depicted in Fig. 1. Staining with Kluver-Barrera (KLB) shows the intense demyelination in the spinal cord (A), optic nerve (B), and brain (C) (bars 500 μm). The positioning of the depicted brain white matter lesion (C) in the corpus callosum is indicated by the rectangle in the insert. The arrowhead in C points at a blood vessel that is further magnified in F and I. CD3 staining shows the presence of inflammation in the spinal cord (D), optic nerve (E), and brain (F) (bars 50 µm). The spinal cord was also stained with anti-PLP (G, bar 100 μm), and the optic nerve was stained for macrophages with MRP14 (H, bar 100 μm). The insert in H shows PLP degradation products in macrophages. The corpus callosum stained with MRP14 for phagocytic macrophages (I, bar 100 μm). Cortical demyelination was verified by PLP staining (J, bar 200 μm), and the activated macrophages/microglia at the border of demyelination were visualized by staining with MRP14 (K, 200 μm). Staining for CD3 shows the presence of a large amount of T cells in the meninges and at the active border of demyelination (L, 200 μm).

are the same as for human or rodent tissue. Briefly, spleens and lymph nodes obtained at necropsy are snap-frozen in liquid nitrogen and stored in −80°C. Frozen spleen and lymph node sections of 6 μm are cut and thaw-mounted on gelatin/chrome alum-coated glass slides. Slides are kept overnight at RT in a humidified atmosphere. After air-drying of the slides for 1 h, they are fixed with fresh acetone containing 0.02% (v/v) H_2O_2. Acetone-fixed slides are air-dried for 10 min and subsequently washed in PBS. Tissue sections are incubated with primary Ab overnight at 4°C in a humidified atmosphere. Incubations with secondary and tertiary reagents are performed for 1 h at RT. Between incubation steps, the slides are washed twice with PBS. This is followed by incubation with horseradish peroxidase (HRP)-labelled avidin-biotin complex (Vectastain ABC, Vector Laboratories, Burlingame, CA, USA). HRP activity is revealed by incubation for 10 min at RT with 3-amino-

9-ethyl carbazole (AEC; Sigma, Zwijndrecht, The Netherlands), leading to a bright red precipitate. Incubation with isotype-matched primary Ab of irrelevant specificity and omission of the primary Ab serve as negative controls.

Concluding Remarks

For decades, the marmoset has been used as an experimental model in biomedical research, e.g., neuroscience, toxicology, reproductive biology, and infectious disease [12, 18]. This publication provides an overview of the techniques and cross-reacting reagents that have been developed for the marmoset over the last 10 to 15 years, and especially for examination of the EAE and CIA models. However, there is still enough space for improvement or for development of new techniques. A crucial point would be the development of serological and molecular techniques to detect (latent) herpesvirus infections, such as for Epstein-Barr virus and cytomegalovirus.

A significant step has been made in refining the animal model for MS, and major improvements have been made at several levels, i.e., of cellular isolation, flow cytometry, and molecular biology. This enables us to answer more research questions with less material and makes the marmoset therefore an excellent model to bridge the gap between rodents and humans.

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