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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
*master in de biomedische wetenschappen: klinische
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Masterproef

The role of collapsin response mediator protein 2 in multiple sclerosis-related
T cell functions

Promotor :
dr. Katrien BUSSCHOTS

Anuschka Vangrinsven

*Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische
wetenschappen, afstudeerrichting klinische moleculaire wetenschappen*

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Preface

This thesis is the result of my senior internship, which I have performed at the Biomedical Research Institute (BIOMED) at the University of Hasselt during the last 8 months. Although not everything went according to plan, I do not regret my choice for a moment. Research is all about teamwork and to get to this point, numerous people helped me. Therefore, I would like to take the opportunity to extend my sincere gratitude to these people.

First of all, I would like to thank Annelies Vanheel, my daily supervisor. Thank you for your guidance and support, but also for sharing your tips and tricks with me. Thank you for letting me actively participate in the planning and pursuance of our experiments and your trust in me. I would also like to thank you for your enduring optimism and all the other work you have done to realize this project.

Moreover, I would like to thank my promotor dr. Katrien Busschots. Thank you Katrien, for giving me the opportunity to perform my senior internship at BIOMED and for your valuable research insights and advice during the meetings.

A word of appreciation also goes to Karen Geunes and Leen Timmermans. Thank you both for all the efforts you have made to help me realize this project, especially during my last experiment in which nothing seemed to go as planned. I would also like to thank Prof. dr. Jean-Paul Noben and Erik Royackers. Thank you for helping me with the mass spectrometric analysis of my samples.

Finally I would like to thank my fellow students and family. Jessica, Nazim, Manolo and Jasmine we have shared a lot of funny moments, but also some frustrations. Bjorn and Elien, these last 5 years would not have been the same without you. I would like to thank my parents and brother for always believing in me and supporting me! Although the last year was not the easiest one, we pulled through in the end. Brecht, I wish you all the luck in realizing your American dream! I thank the rest of my family for their interest in my studies. Thank you for your support!

List of abbreviations

APC	Antigen presenting cell
CaI	Calcium-ionophore
CNS	Central nervous system
CRMP2	Collapsin response mediator protein 2
CCL2	Chemokine (C-C motif) ligand 2
CXCL12	Chemokine (C-X-C motif) ligand 12
DAPI	4',6-Diamidino-2-phenylindole
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemoluminescence
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GSK3 β	Glycogen synthase kinase 3 beta
HRP	Horseradish peroxidase
IFN γ	Interferon gamma
IL	Interleukin
MFI	Mean fluorescence intensity
MS	Multiple sclerosis
NP40	Nonidet-P40
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing-remitting multiple sclerosis
siRNA	Small-interfering RNA
SPMS	Secondary progressive multiple sclerosis
TGF β	Transforming growth factor beta
T _H cell	T helper cell
TNF α	Tumor necrosis factor alpha
Treg	Regulatory T cell

Summary

Background: Multiple sclerosis (MS) is a demyelinating autoimmune disease of the central nervous system (CNS) in which T cells play an important role. However, little is known about the underlying mechanisms of this disease. Recently, a proteomics study performed in our research group illustrated the differential expression of collapsin response mediator protein 2 (CRMP2) in experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Additionally, it was shown in literature that there are increased levels of phosphorylated CRMP2 present in chronic MS lesions and that CRMP2-expressing peripheral T cells show a higher migration rate into the CNS during virus-induced neuroinflammation. These findings could indicate a role for CRMP2 in MS pathology.

Objectives and results: We hypothesize that CRMP2 knockdown results in an altered migration rate and differentiation capacity of T cells, which could improve the clinical outcome in MS and EAE. To test this hypothesis, an *in vitro* experiment was set up, in which Jurkat T cells and CD4⁺ primary T cells were used. Because, T cells are rather difficult to transfect, the transfection efficiency needed to be optimized. Three different aspects of transfection were explored: the transfection method, the effect of different culture media and the effect of activation on the transfection efficiency. The highest transfection efficiency was obtained when activated cells were transfected using the TransFectin™ lipid reagent in serum-reduced conditions. Next, we wanted to establish a knockdown of CRMP2 in both the Jurkat and the CD4⁺ primary T cells. Both 120 and 144 hours after transfection a knockdown of CRMP2 could be established in the Jurkat T cells (a knockdown of 21 and 63% respectively).

Finally, we wanted to investigate the effect of CRMP2 knockdown on T cell migration and differentiation. Due to time limitations, these T cell properties could not be explored. Future perspectives not only include the exploration of these T cell properties, but also the effect of CRMP2 knockdown in adoptive transfer EAE will be explored.

Samenvatting

Achtergrond: Multiple sclerose (MS) is een demyeliniserende auto-immuun ziekte van het centrale zenuwstelsel, waarin T cellen een belangrijke rol spelen. Over de onderliggende ziektemechanismen is er nog steeds weinig geweten. Een proteomica study, die werd uitgevoerd in onze onderzoeksgroep, heeft de differentiële expressie aangetoond van collapsin response mediator protein 2 (CRMP2) in het diermodel van MS. Daarnaast, werd er in de literatuur reeds aangehaald dat er verhoogde gehalten gefosforyleerd CRMP2 aanwezig zijn in chronische MS lesies en dat een hogere expressie van CRMP2 in perifere T cellen gepaard gaat met een hogere migratie van deze T cellen naar het CZS tijdens virus-geïnduceerde neuroinflammatie. Samen wijst dit op een mogelijke rol voor CRMP2 in MS.

Doel en resultaten: We veronderstellen dat een knockdown van CRMP2 zal resulteren in een gewijzigde migratie en differentiatie van T cellen, die gunstig kan zijn in MS en het diermodel van MS. Om dit te onderzoeken, werd er een *in vitro* experiment uitgevoerd, waarin er gebruik werd gemaakt van zowel humane Jurkat T cellen als CD4⁺ primaire T cellen. Omdat T cellen moeilijk te transfecteren zijn, was het in eerste instantie nodig om de transfectie efficiëntie te optimaliseren. Hierbij werd er vooral aandacht besteed aan de transfectie methode, het effect van verschillende kweekmedia en het effect van activatie op de transfectie efficiëntie. De hoogste transfectie efficiëntie werd bereikt wanneer geactiveerde cellen werden getransfecteerd met behulp van TransFectine in serum-reduceerde omstandigheden. Het volgende doel was het verkrijgen van een knockdown van CRMP2 in zowel Jurkat als de CD4⁺ primaire T cellen. Zowel 120 uur als 144 uur na de transfectie kon er een daling in de CRMP2 expressie geobserveerd worden in de Jurkat T cellen. Na 120 uur was er een knockdown van 21% in de CRMP2 expressie en na 144 uur was er een knockdown van 63%.

Vervolgens, wouden we het effect van een knockdown van CRMP2 op T cel migratie en differentiatie onderzoeken. Deze T cel eigenschappen konden wegens een gebrek aan tijd niet meer onderzocht worden. Daarom zal in toekomstig onderzoek allereerst het effect van een knockdown van CRMP2 worden onderzocht op deze T cel functies. Dit brengt ons een stapje dichterbij ons uiteindelijke doel waarbij we het effect van de knockdown zullen bestuderen in een adoptieve transfer diermodel van MS.

1 Introduction

Multiple sclerosis (MS), one of the leading causes of neurological deficits in young adults, is a chronic inflammatory disease of the central nervous system (CNS) which affects up to 2.5 million people worldwide [1-3]. The first symptoms of MS usually develop between the age of 20 and 40 and women are approximately 3 times more prone to develop this disease than men [4]. Regarding the course of the disease, three important subtypes can be distinguished, namely relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). Approximately 85% of the MS patients have RRMS, which is characterized by the alternating occurrence of neurological deficits (relapses) and periods of remission. Most of these RRMS patients (40%) will develop SPMS in time, which is characterized by an increase in neurodegeneration without the presence of recovery periods. In the last subtype of MS, namely PPMS (15% of the MS patients), the disease is progressive from the beginning [3, 5]. Although the etiology of this disease is still unknown, it is believed that the development of MS is probably dependent on both genetic (e.g. alleles of interleukin-7 receptor A, interleukin-2 receptor A and in the human leukocyte antigen locus) and environmental (e.g. smoking, vitamin D, viruses) factors [6, 7]. Besides this, it is assumed that the immune system plays a critical role.

Most of the knowledge regarding the pathogenesis of MS is based on studies performed in the animal model of MS, namely experimental autoimmune encephalomyelitis (EAE). Because there is a high similarity between the EAE model and MS regarding the pathologic and clinical properties, this animal model can help to get a better understanding of the processes that play a role in MS [4, 8, 9].

1.1 Immunopathogenesis of MS

Upon activation of auto-reactive T cells in the periphery by antigen-presenting cells (APC), these T cells will express adhesion molecules (e.g. intercellular adhesion molecule 1 and vascular cell adhesion protein 1) and proteases (e.g. matrix metalloproteinases), which allows them to cross the blood-brain barrier. After reactivation of the T cells in the CNS by local APCs (e.g. microglia) they will recruit other immune cells into the CNS and trigger an inflammatory cascade by the production of pro-inflammatory molecules e.g. cytokines and nitric oxide, which eventually causes the breakdown of myelin, axonal damage and the loss of oligodendrocytes (Figure 1)[9, 10]. Nevertheless it remains rather indefinite how these auto-reactive T cells become activated in the first place. A possible explanation for the activation of these T cells is molecular mimicry, a process in which cross-reactivity between foreign peptides (e.g. microbial or viral peptides) and self-peptides (e.g. myelin peptides)

occurs. Another activation theory, the so-called bystander activation includes the activation by superantigens, inflammatory cytokines and toll-like receptor-triggered infection [11, 12].

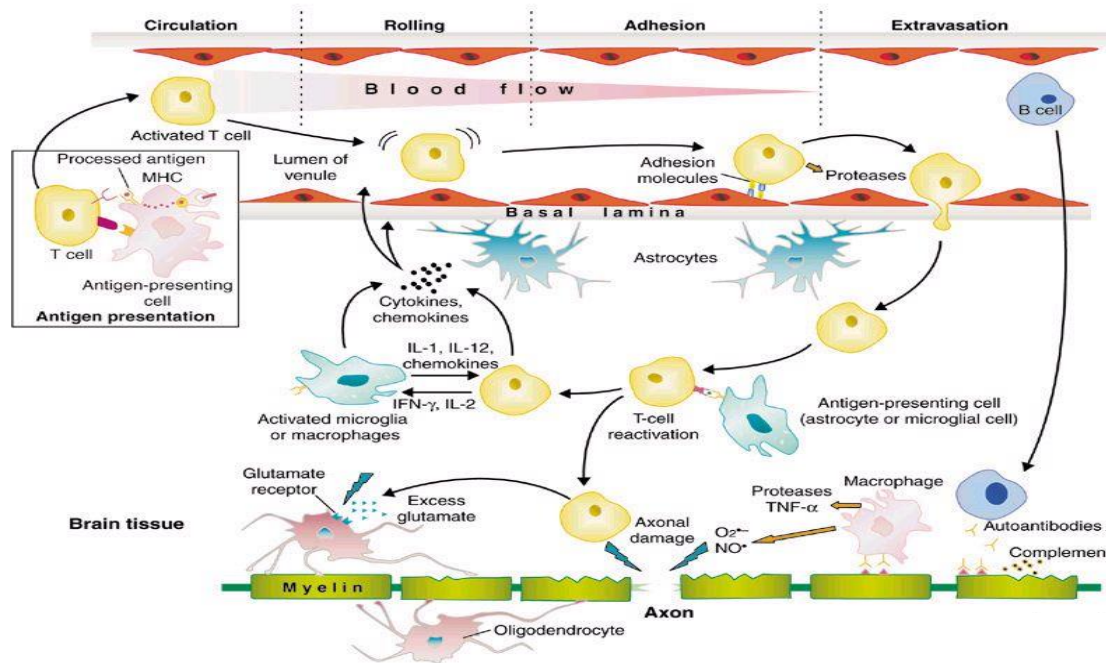


Figure 1: Schematic overview of the role of T cells in multiple sclerosis. Peripheral T cells become activated by APCs. These T cells will then express adhesion molecules and proteases, which allows them to cross the blood-brain barrier. In the CNS, the T cells become reactivated by local antigen presenting cells. This leads to the recruitment of other immune cells into the CNS and to an inflammatory cascade, which ultimately causes the breakdown of myelin, axonal damage and the loss of oligodendrocytes. (APC: antigen-presenting cell, CNS: central nervous system, IL: Interleukin, IFN: interferon, NO: nitric oxide, $O_2^{\cdot-}$: superoxide, TNF: tumor necrosis factor, MHC: major histocompatibility complex) [13]

T cells can be divided into $CD8^+$ and $CD4^+$ T cells, of which the latter can be further subdivided into four groups regarding the cytokines they produce upon activation. These four groups are: (1) T helper (T_H) 1 cells, (2) T_H2 cells, (3) T_H17 cells and T regulatory cells (Tregs). T_H1 cells which produce e.g. the pro-inflammatory cytokines interferon gamma ($IFN\gamma$) and tumor necrosis factor alpha ($TNF\alpha$), originate from naïve T cells (T_H0 cells) by the production of interleukin (IL) 12 by APCs. T_H2 cells on the other hand produce anti-inflammatory cytokines e.g. IL-4, IL-5, IL-13. This class of $CD4^+$ T cells derive from naïve T cells by the production of IL-4 by APCs. The third class of T cells, namely the IL-17 producing cells are termed T_H17 cells. These cells originate from naïve T cells under the production of IL-6 and transforming growth factor beta ($TGF\beta$). When naïve T cells encounter $TGF\beta$ -producing APCs, Tregs arise, which are known to produce $TGF\beta$. The $CD8^+$ T cells, on the other hand, are best known from their direct cytotoxic activities towards MHC class I-expressing cells. Cells that express MHC class I under inflammatory conditions are e.g. neurons, astrocytes and oligodendrocytes [14].

T cells play a significant role in MS pathology. In MS lesions, both CD4⁺ and CD8⁺ T cells could be detected. The CD4⁺ cells are found in the perivascular cuffs, whereas the CD8⁺ T cells are mostly present in the center and the border zone of the lesion [14]. Several studies show that there are similar amounts of myelin-reactive T cells present in the periphery of MS patients and healthy controls, but there is a difference regarding the activation status of these T cells. It was found that the T cells of MS patients were activated (expression of the IL-2 receptor), which was not the case in the healthy controls. It was also observed that the auto-reactive T cells of MS patients had a Th1 phenotype, which could point to a pathogenic role for these T cells [10, 11, 15].

In spite of this knowledge, there still is a poor understanding of the underlying disease mechanisms of MS (e.g. the initial trigger that activates the auto-reactive T cells). To improve the development of more efficient therapies, it is necessary to gain more insight into these underlying mechanisms.

1.2 Collapsin response mediator protein 2

Recently, researchers of the Biomedical Research Institute (Diepenbeek) attempted to explore these underlying disease mechanisms of MS by the use of a proteomics study. The study revealed 75 proteins, which were differentially expressed in EAE and the phosphoprotein collapsin response mediator protein 2 (CRMP2) was one of them. CRMP2 was present in 10 differential spots (6 upregulated and 4 downregulated). A difference in the molecular weight in the two-dimensional difference in-gel electrophoresis indicated the presence of e.g. isoforms and breakdown products, whereas the same molecular weight pointed to e.g. post-translational modifications [16].

CRMP2 is one of the five members of the collapsin response mediator protein family. There are two subtypes of CRMP2, namely CRMP2A (75 kDa) and CRMP2B. The latter includes several cytosolic isoforms with molecular weights between 62 and 66 kDa, and one shorter isoform with a molecular weight of 58 kDa [17, 18]. CRMP2 is best known as a mediator of semaphorin 3 (collapsin) which has a chemorepellent action on axonal growth [18]. The phosphorylation of CRMP2 by glycogen synthase kinase 3 beta (GSK3 β) at Thr-509/514 results into CRMP2 inactivation and consequently into a decline in neuronal polarization. Inhibition of GSK3 β , in contrary, leads to an increased CRMP2 activity and to axonal outgrowth and branching [19].

Menon *et al.* observed elevated levels of phosphorylated CRMP2 in chronic MS lesions, which also indicates the neural expression of CRMP2. More precisely, phosphorylated CRMP2 could be detected in the brain and spinal cord of patients with active MS lesions, whereas it could not be detected in control samples. Also in the animal model of MS, EAE, there were increased levels of phosphorylated

CRMP2 present, especially at the peak of disease [20, 21]. Petratos *et al.* also described a role for CRMP2 in neurons during MS pathology [21].

Besides the neural role for CRMP2, this protein is important in T cell polarization and consequently migration. Vincent *et al.* discovered that CRMP2 is able to interact with vimentin, a cytoskeletal element, which implies a role for CRMP2 in the cytoskeleton rearrangement that takes place during the polarization of T cells. CRMP2-silencing resulted in a decreased T cell polarization and migration confirming the involvement of CRMP2 in these two processes. T cell polarization and migration were promoted in the case of CRMP2 overexpression [22, 23]. Chemokines e.g. chemokine (C-X-C motif) ligand 12 (CXCL12) can cause T cell polarization and consequently migration through the signaling cascade that starts upon the interaction of the chemokine with its receptor. CRMP2 is involved in this process. The interaction of CXCL12 with its receptor causes a decrease in the Thr-509/514 phosphorylation by GSK3 β and Ser-522 by cyclin-dependent kinase-5, whereas the phosphorylation of CRMP2 Tyr-479 by Yes kinase increases (Figure 2). The phosphorylation at Tyr-479 results in cytoskeletal rearrangement and the migration of T cells [24].

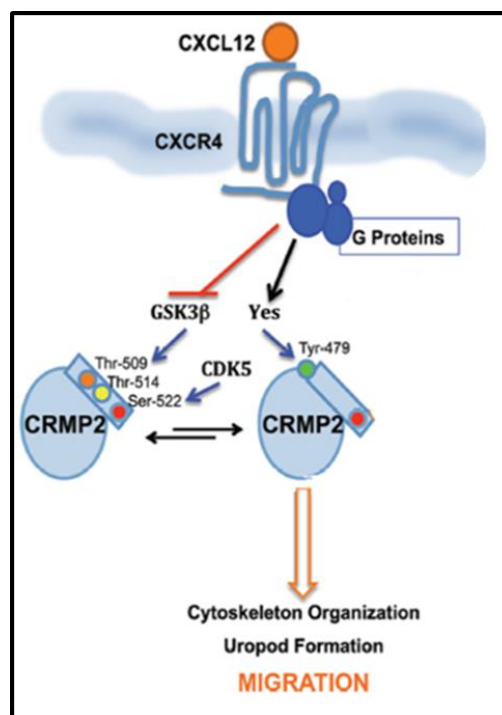


Figure 2: The effect of CXCL12 treatment on CRMP2 activity. Interaction of CXCL12 with its receptor, CXCR4 causes phosphorylation of CRMP2 on Tyr-479, which results in cytoskeletal rearrangement and migration of T cells. The interaction also results in a decreased phosphorylation at Thr-509/514 and Ser-522 of CRMP2. (CXCL12: chemokine [C-X-C motif] ligand 12; CXCR4: C-X-C chemokine receptor type 4; GSK3 β : glycogen synthase kinase 3 beta; CRMP2: collapsin response mediator protein 2; Cdk5: cyclin dependent kinase 5). Adapted from: [24].

The involvement of CRMP2 in the migration of T cells was also observed during virus-induced neuroinflammation. Vuillat *et al.* illustrated that the expression of CRMP2 in peripheral T cells correlates with the migration of these cells into the CNS during virus-induced neuroinflammation [25].

1.3 Study aims and experimental design

A proteomics study performed in our research group illustrated the differential expression of CRMP2 in EAE rats [16]. In literature, it is already shown that there is a neural expression of CRMP2 in MS and that elevated levels of CRMP2 in T cells correlate with a higher migration rate into the CNS during other neuroinflammatory diseases [20, 25]. These findings indicate a role for CRMP2 in MS.

In this study, we hypothesize that CRMP2 knockdown in MS results in an altered migration rate and differentiation capacity of T cells. To test this hypothesis an *in vitro* experiment will be set up. Because T cells are difficult to transfect, the transfection efficiency must be optimized. We will optimize for three different aspects of transfection by means of a fluorescent-siRNA. These three aspects are the transfection method, the effect of different culture media and the effect of activation on the transfection efficiency. After each optimization step, fluorescence microscopy and flow cytometry will be used to analyze the transfection efficiency. Next, a knockdown of CRMP2 will be established by means of small-interfering RNAs (siRNAs). To validate the knockdown, T cells treated with the CRMP2-siRNA will be compared to T cells treated with scramble-siRNA. The knockdown will be validated on different time points by the use of western blotting.

In future experiments, we will compare the functional properties between the T cells transfected with CRMP2-siRNA and those transfected with scramble-siRNA, both *in vitro* and *in vivo*. The functional properties that will be investigated are T cell migration and T cell differentiation. By performing all these experiments, we hope to clarify the role of CRMP2 in MS-related T cell functions.

2 Materials and methods

2.1 Cell culture

Jurkat cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% fetal calf serum (FCS) (Gibco), 0.5% penicillin/streptomycin, 1% non-essential amino acids and 1% sodium pyruvate (Sigma-Aldrich). The cells were cultivated at 37°C in an atmosphere of 5% CO₂ and were passaged two times a week, initial density approximately 10.10⁶ cells per ml.

2.2 Isolation of CD4⁺ primary T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using lympholyte human cell separation media (Cedarlane). CD4⁺ primary T cells were isolated from the PBMCs by means of the EasySep human CD4 positive selection kit (StemCell Technologies) following the manufacturer's instructions. Briefly, a mononuclear cell suspension of 1.10⁸ cells/ml was prepared in EasySep buffer. The cells were incubated for 15 minutes with EasySep positive selection cocktail (100µl/ml) and for 10 minutes with magnetic nanoparticles (50µl/ml). The positively selected cells, which were obtained using a magnet (StemCell Technologies), were counted by means of a Fuchs-Rosenthal counting chamber and seeded in a 6-well plate at 2.10⁶ cells/2 ml.

2.3 CRMP2 knockdown

The transfection efficiency of Jurkat cells was explored by means of a fluorescein-siRNA transfection control, after which the cells were transfected with CRMP2-siRNA.

2.3.1 Fluorescent transfection control

Jurkat cells were activated for 5 days with CD3/CD28 human T-activator Dynabeads (Invitrogen) and seeded at 8.10⁵ cells/well in 0.5 ml culture medium the day before transfection (day 4 of activation). Just before transfection, the cells were resuspended in Opti-MEM medium (Gibco). Next, the cells were transfected with fluorescein-siRNA transfection control (New England Biolabs) or Block-it[™] fluorescent oligo (Invitrogen) using TransFectin[™] lipid reagent (BIO RAD) according to the instructions of the manufacturer. After incubation with the fluorescein-siRNA transfection control, the transfection efficiency of the cells was determined by means of flow cytometric analysis by using the FACS caliber flow cytometer (BD biosciences). For this, the cells were centrifugated and the pellet

was resuspended in FACS buffer (1 x phosphate buffered saline (PBS), 2% FCS and sodium azide). Besides the flow cytometric analysis, also microscopic analysis was performed by means of the Axiovert 100 (Jena optics).

2.3.2 CRMP2-siRNA

Jurkat cells were transfected with CRMP2-siRNA (sequence see Table 1) (Eurogentec) using TransFectin™ lipid reagent (BIO RAD). siRNA-scramble (sequence see Table 1)(Eurogentec) was used as a negative control. The knockdown of CRMP2 was validated by means of western blotting.

Table 1: siRNA sequences

siRNA	Sequence
CRMP2-siRNA (5' → 3')	AAG-AUG-GGU-UGA-UCA-AGC-AAA-55
CRMP2-siRNA reverse (5' → 3')	UUU-GCU-UGA-UCA-ACC-CAU-CUU-55
siRNA-scramble (5' → 3')	GAA-ACA-GAC-GAG-UAG-GAU-AUU-55
siRNA-scramble reverse (5' → 3')	AAU-AUC-CUA-CUC-GUC-UGU-UUC-55

2.4 Protein isolation

Jurkat cells and CD4⁺ primary cells were washed with PBS and lysed with Nonidet-P40 (NP40) buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP-40 and protease inhibitor cocktail). For the lysis of 8.10⁵ cells, 50 µl of NP40 buffer was used. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo scientific) and the lysates were stored at -20°C.

2.5 Western blotting

The isolated proteins (10 µg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis, after which they were blotted onto a nitrocellulose membrane. Next, the membrane was incubated overnight with the primary antibody rabbit anti-CRMP2 (1/1000, Cell signaling technology), after which the membrane was incubated with the secondary antibody goat anti-rabbit horseradish peroxidase (HRP) for 2 hours (1/2000, Dako). The expression of CRMP2 was detected by enhanced chemoluminescence (ECL) by means of the Pierce ECL plus western blotting substrate (Thermo scientific) according to the manufacturer's instructions. B-actin was used as a loading control. After an overnight incubation of the membrane with the primary antibody mouse

monoclonal IgG₁ β-actin (1/5000, Santa Cruz Biotechnology), the membrane was incubated with the secondary antibody polyclonal rabbit anti-mouse immunoglobulins/HRP (1/5000, Dako) for 1 hour.

2.6 Immunohistochemistry

Spinal cord cryosections (10 μm) of acute EAE rats were used for the detection of CRMP2 and T cells in MS lesions. To detect CRMP2 and CD3 the sections were incubated overnight with rabbit anti-CRMP2 (1/500, Abcam) and mouse anti-rat CD3 biotin (1/15, AbD serotec) as primary antibodies. Next, the sections were incubated with the secondary antibodies for 1.5 hours, namely Alexa fluor 488 goat anti-rabbit IgG (1/250, Invitrogen) and Alexa fluor 555 streptavidin (1/2000, Invitrogen). 4',6-Diamidino-2-phenylindole (DAPI) was used as a counterstaining. The Nikon ECLIPSE 80i microscope and the NIS elements software were used to analyze the immunostaining.

3 Results

In literature it was already shown that there are elevated levels of phosphorylated CRMP2 present in chronic MS lesions and that an elevated expression of CRMP2 in peripheral T cells results in an enhanced migration of these cells into the CNS during virus-induced neuroinflammation [20, 21, 25]. These findings, together with the results of a proteomics study in which the differential expression of CRMP2 was demonstrated in EAE rats, could indicate a role for CRMP2 in MS [16]. We hypothesized that CRMP2 knockdown in T cells results in an altered migration rate and differentiation capacity of these cells.

3.1 CRMP2 expression in T cells migrated to MS lesions

A differential expression of CRMP2 was already observed in the brainstem of EAE rats and it was already shown that CRMP2 plays a role in T cell function during other neuroinflammatory diseases. Because T cells play an important role in MS, we wanted to investigate the expression of CRMP2 in T cells infiltrated into EAE lesions by means of a fluorescent staining for CRMP2 and CD3 on spinal cord cryosections of EAE rats. In the negative control, no staining of the secondary antibodies was observed (Figure 3A). Both CRMP2 and CD3 were found in the MS lesion (Figure 3B and C). The results also showed co-localization of the CRMP2 and CD3 signal (Figure 3C), which indicates that CRMP2 is expressed in T cells which are migrated to MS lesions.

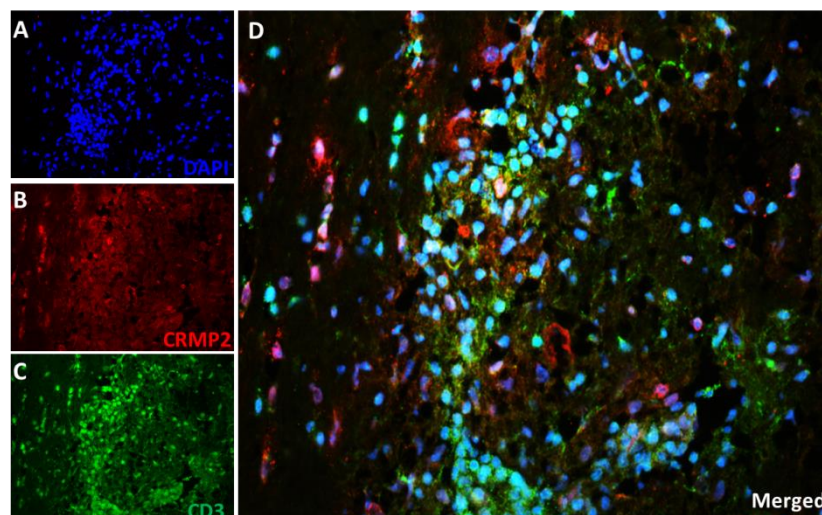


Figure 3: The presence of CRMP2 in T cells migrated to MS lesions. Fluorescent staining of the negative control (A), CRMP2 (B) and CD3 (C) in the MS lesion. Fluorescent double staining of CRMP2 and CD3 in the MS lesion (D). Pictures were taken with a 40x objective. (DAPI: 4',6-Diamidino-2-phenylindole)

3.2 Verification of the CRMP2 expression in T cells

The differential expression of CRMP2 during EAE and the effect of CRMP2 on T cell function during other neuroinflammatory disorders, led us to investigate the effect of CRMP2 in MS-related T cell functions. This was done by means of an *in vitro* experiment, in which human Jurkat T cells and CD4⁺ primary T cells were used. Western blotting was performed to confirm the expression of CRMP2 in these cells. The results showed that CRMP2 is present in both the human Jurkat T cells and primary CD4⁺ T cells (Figure 4). The expression of CRMP2 was also verified by means of mass spectrometry (data not shown).

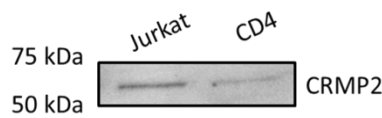


Figure 4: Expression of CRMP2 in Jurkat cells and CD4⁺ primary T cells. Western blot analysis of CRMP2 expression in protein lysates (10 µg) from Jurkat cells and CD4⁺ primary T cells.

3.3 Optimization of the transfection efficiency

To determine the most optimal transfection conditions, Jurkat cells were used to optimize the transfection efficiency. This cell line was used instead of the CD4⁺ primary T cells to limit the amount of blood samples although fine-tuning is necessary for all different cell types. The transfection efficiency of the Jurkat cells was optimized by means of the fluorescein-siRNA transfection control (New England Biolabs) or the Block-it[™] Fluorescent OLIGO (Invitrogen), which are fluorescein labeled RNAs that allow us to check the transfection efficiency. Flow cytometry and fluorescence microscopy (Axiovert 100) were used to explore the cells that were fluorescein positive.

3.3.1 Optimization of the transfection method

Three different transfection methods were explored to determine which one yielded the highest transfection efficiency. These three methods were electroporation, TransFectin[™] lipid reagent and INTERFERin[™] siRNA transfection reagent (Polyplus transfection). The electroporation was carried out by means of the ECM 830 electroporator (BTX Harvard apparatus) for which two different protocols were used (see supplementary data). To determine the highest transfection efficiency, different concentrations of fluorescein-siRNA transfection control and transfection reagent were used (Table 2).

Table 2: Concentrations fluorescein-siRNA transfection control and transfection reagent.

Transfection method	Concentration fluorescein-siRNA	Amount transfection reagent
<i>Electroporation</i>	15 nM	No transfection reagent
<i>TransFectin</i>	15 nM	0,25 µl
		2 µl
		4 µl
	100 nM	4 µl
<i>INTERFERin</i>	100 nM	3 µl

The fluorescein-siRNA transfection control was used at a concentration of 15 and 100 nM. The first concentration was the recommended usage concentration (datasheet), whereas the second concentration was depicted from literature [26]. To determine the most optimal amount of TransFectin™ lipid reagent for the transfection of the Jurkat cells, three different amounts were tested (0.25 µl, 2µl and 4 µl). These three amounts were determined by consulting the datasheet of this transfection reagent. The amount of INTERFERin™ siRNA transfection reagent used, was based on literature [26]. In first instance, Jurkat cells were transfected with fluorescein-siRNA transfection control at a concentration of 15 nM using electroporation and the TransFectin™ lipid reagent. Jurkat cells, which were only electroporated and TransFectin-treated (without the fluorescein-siRNA transfection control) were used as a negative control. Microscopic analysis showed that after electroporation there was no fluorescent signal present (Figure 5A), whereas transfection by means of the TransFectin™ lipid reagent showed a clear fluorescent signal when 4 µl of the reagent was used (Figure 5B). Because this fluorescent signal was still rather low, a higher concentration of the fluorescein-siRNA transfection control was tested, namely 100 nM. This concentration was used to transfect Jurkat cells by means of the TransFectin™ lipid reagent (Figure 5C) and INTERFERin™ siRNA transfection reagent (Figure 5D). Electroporation was excluded, because in the previous experiment no transfection had occurred. The results showed that the TransFectin™ lipid reagent yielded the highest transfection efficiency. Jurkat cells treated with only TransFectin or INTERFERin (without the fluorescein-siRNA transfection control) were used as negative controls.

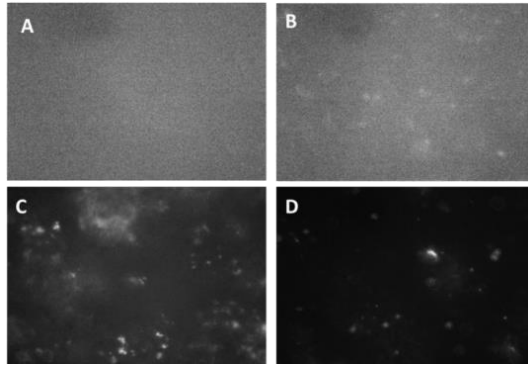


Figure 5: Microscopic analysis of Jurkat cells transfected with fluorescein-siRNA transfected control. Transfection of Jurkat cells with 15 nM fluorescein-siRNA transfected control using electroporation (A) and 4 µl of TransFectin™ lipid reagent (B). Jurkat cells transfected with 100 nM fluorescein-siRNA transfected control using 4 µl of TransFectin™ lipid reagent (C) and 3 µl of INTERFERin™ siRNA transfected reagent (D). The fluorescein isothiocyanate (FITC) channel was used to detect the presence of a fluorescent signal.

To obtain a quantitative measure of the transfection efficiency and to confirm that the fluorescent signal was present in the cells, flow cytometry was used. The forward and sideward light scatter plots were used to gate for the living Jurkat cells (Figure 6A). Next, the presence of a fluorescein signal was explored in these living Jurkat cells by means of the fluorescein isothiocyanate (FITC) channel, which indicated that the cells were effectively transfected with the fluorescein-siRNA transfected control (Figure 6B). As a negative control Jurkat cells were transfected with only the TransFectin™ lipid reagent and the INTERFERin™ siRNA transfected reagent, thus without the fluorescein-siRNA transfected control (Figure 6A and B, on the left).

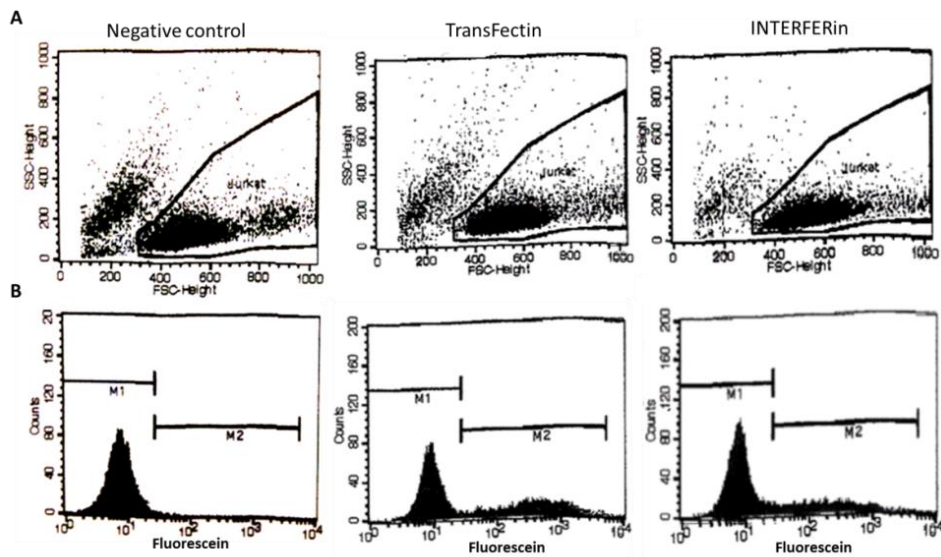


Figure 6: FACS analysis of Jurkat cells transfected with the fluorescein-siRNA transfected control. Forward and sideward light scatter plots (A) and the presence of a fluorescein signal in Jurkat cells transfected using TransFectin™ lipid reagent (middle) and INTERFERin™ siRNA transfected reagent (right). Jurkat cells which were only TransFectin-treated (without the fluorescein-siRNA transfected control) were included as an example of a negative control (B, on the left).

The percentage living cells was approximately 90% for both transfection methods (Figure 7A). The Jurkat cells which were transfected using the TransFectin™ lipid reagent had a transfection efficiency of approximately 35%, whereas only 21.5% of the cells transfected by means of the INTERFERin™ siRNA transfection reagent were fluorescein positive (Figure 7B). Besides the percentage fluorescein positive cells, the mean fluorescence intensity (MFI) of the cells was used as a measure of the transfection efficiency. The higher the MFI, the higher the amount of fluorescein-siRNA transfection control present in the cells. The MFI was the highest when the Jurkat cells were transfected using the TransFectin™ lipid reagent (MFI = ± 635) (Figure 7C). The results indicated that when Jurkat cells are transfected using the TransFectin™ lipid reagent, both the transfection percentage and the MFI were higher compared to the INTERFERin™ siRNA transfection reagent. Thus, the transfection efficiency is the highest when cells are transfected using the TransFectin™ lipid reagent. Therefore, in the following experiments the Jurkat cells will be transfected using this reagent.

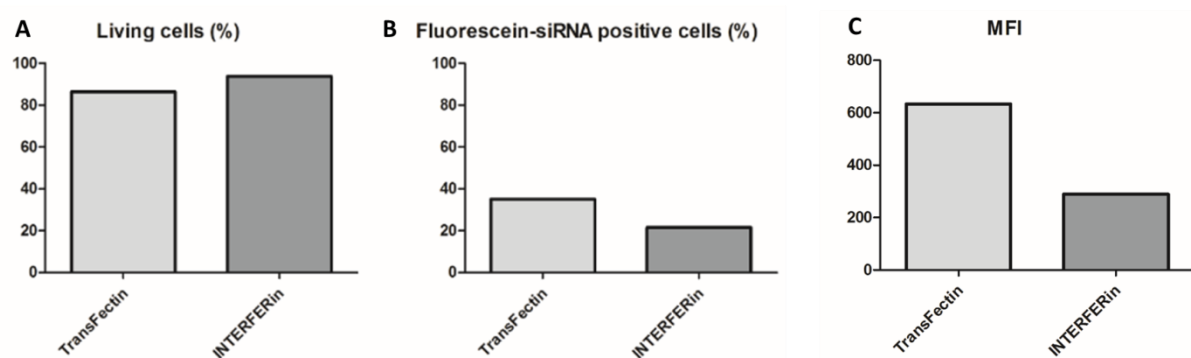


Figure 7: Effect of different transfection methods on the transfection efficiency of Jurkat cells. The percentage of living cells and fluorescein-siRNA positive cells, and the MFI were compared between the two transfection methods (TransFectin™ lipid reagent and INTERFERin™ siRNA transfection reagent). (MFI: mean fluorescence intensity).

3.3.2 Effect of different culture media on the transfection efficiency

In literature, transfections are often performed in serum-reduced conditions [26]. This is the reason why three different culture media were explored. The media that were used are complete medium, RPMI 1640 and Opti-MEM. Jurkat cells which were only treated with the TransFectin™ lipid reagent (without the fluorescein-siRNA transfection control) were used as a negative control. To determine which medium resulted in the highest transfection efficiency flow cytometry was used. Flow cytometric analysis indicated that the percentage living cells was more or less the same between the different conditions (87-90%)(Figure 8A). The percentage fluorescein positive cells (Figure 8B) and the MFI (Figure 8C) were the highest when Opti-MEM medium was used as culture medium during transfection. Thus, for further experiments, this culture medium will be used.

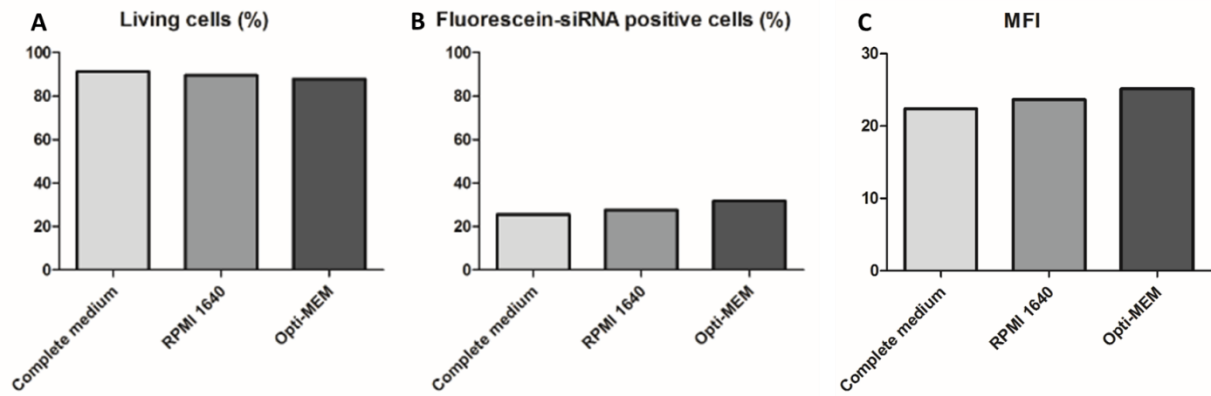


Figure 8: Effect of different culture media on the transfection efficiency of Jurkat cells. The percentage living cells (A), fluorescein-siRNA positive cells (B) and the MFI (C) after transfection with the fluorescein-siRNA transfection control using the TransFectin™ lipid reagent. (MFI: mean fluorescence intensity).

3.3.3 Effect of activation on transfection efficiency

For future experiments, which include an adoptive transfer EAE, it was important to explore whether activation of the T cells resulted in a changed transfection efficiency. In adoptive transfer EAE, activated myelin-specific T cells of an immunized animal will be injected into a healthy recipient [9]. To determine the effect of activation, the transfection efficiency between Jurkat cells with and without activation was compared. The cells were either activated with phorbol 12-myristate 13-acetate (PMA) and calcium-ionophore (Cal) or with CD3/CD28 human T-activator Dynabeads. Different time points of activation were used (3, 4 and 5 days). Jurkat cells that were transfected by the use of the TransFectin™ lipid reagent, but without fluorescein-siRNA transfection control were used as a negative control. After transfection of the cells with the fluorescein-siRNA transfection control, microscopic and flow cytometric analysis were performed.

Microscopic analysis showed that both the Jurkat cells with and without activation were fluorescein positive (Figure 9B-E). In the negative control (without the fluorescein-siRNA transfection control) there was no fluorescent signal present (Figure 9A).

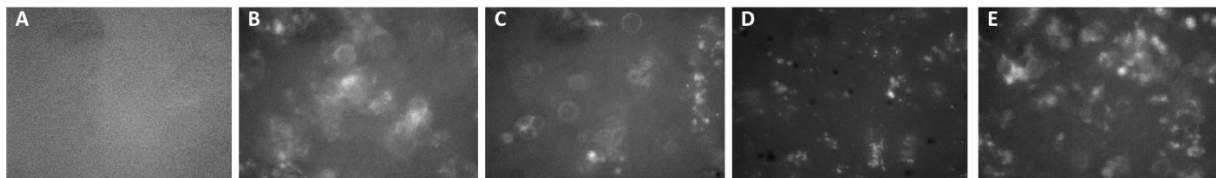


Figure 9: Microscopic analysis of Jurkat cells with and without activation. Transfection without the fluorescein-siRNA transfection control (A) and with the fluorescein-siRNA transfection control of (B) not-activated, (C) 3 days, (D) 4 days and (E) 5 days CD3/CD28 activated Jurkat cells using TransFectin™ lipid reagent.

Flow cytometric analysis showed that Jurkat cells which were activated using PMA and Cal had an increased percentage of death cells (8,8% living cells; data not shown). This high amount of cell death indicated that the other results (percentage fluorescein-siRNA positive cells and the MFI) obtained after the activation of the cells with PMA and Cal were not reliable. The results of the Jurkat cells activated with the CD3/CD28 beads (3, 4 and 5 days) showed that activation resulted in a minor increase in the percentage living cells (Figure 10A) and fluorescein positive cells (Figure 10B). After 5 days of activation, the percentage fluorescein positive cells was approximately 60%. Activation of the Jurkat cells caused a decrease in the MFI (Figure 10C). This decrease was rather small when the Jurkat cells were activated during 5 days. Because, the percentage living cells and fluorescein positive cells was the highest when the Jurkat cells were stimulated during 5 days, and because there was only a minor decrease in the MFI after 5 days of activation, this condition was used in the following experiments.

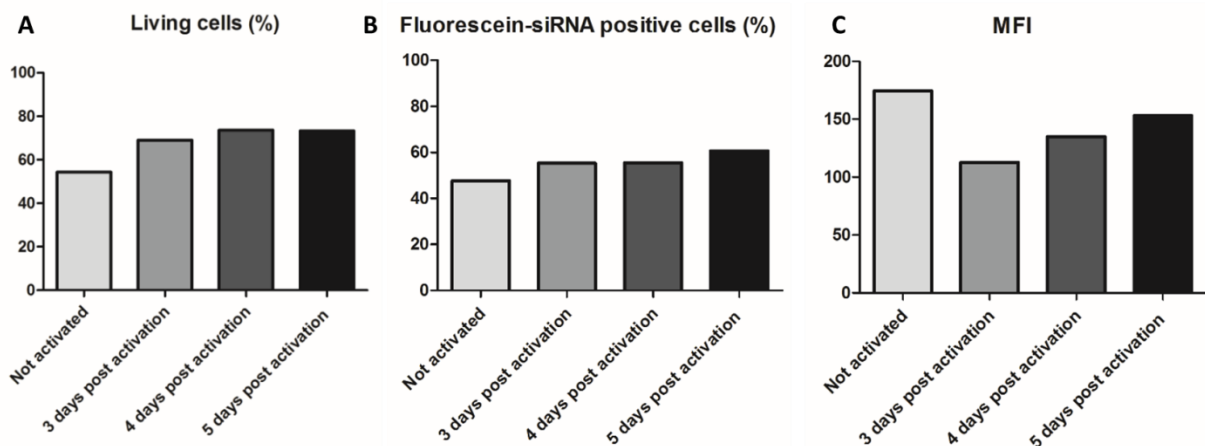


Figure 10: Effect of activation on the transfection efficiency of Jurkat cells. Comparison of Jurkat cells with and without activation. The cells were activated during different time points (3, 4 and 5 days) with CD3/CD28 beads. The percentage living cells (A), fluorescein-siRNA positive cells (B) and the MFI (C) after transfection with the fluorescein-siRNA transfection control using the TransFectin™ lipid reagent. (MFI: mean fluorescence intensity).

3.4 CRMP2 knockdown of Jurkat cells

The differential expression of CRMP2 in EAE rats, together with the effect of CRMP2 on T cell function during other neuroinflammatory diseases, led us to hypothesize that CRMP2 knockdown in MS T cells could result in an altered migration and differentiation of T cells. To test this hypothesis it was necessary to obtain a knockdown of CRMP2. This knockdown was established by means of siRNAs and the conditions determined in the previous part. To determine the percentage of knockdown, the CRMP2 expression was compared between Jurkat cells transfected with CRMP2-

siRNA and with scramble-siRNA. The CRMP2-siRNA and scramble-siRNA were already described in literature [22].

Two different concentrations of siRNA were used for the transfection of the Jurkat cells, namely 67 nM and 100 nM. The latter corresponds with the concentration of fluorescein-siRNA transfection control used in the previous experiments. The lower concentration, which was exactly 1 μ l less than when the 100 nM was used, was included to determine whether this concentration could already result in a knockdown or not. There were also two types of controls included in the experiment. Jurkat cells transfected with scramble-siRNA were used as a negative control, and Jurkat cells transfected with the Block-itTM fluorescent oligo were used as a positive control for transfection. This fluorescent control was used instead of the fluorescein-siRNA transfection control (previous experiments), because the latter was no longer being manufactured. This positive control was used to determine whether the siRNA was efficiently delivered into the Jurkat cells. To explore the delivery of the fluorescent siRNA into the cells, flow cytometry and fluorescence microscopy was used. The percentage fluorescein positive cells was approximately 36% and the MFI was approximately 900, which indicates that transfection had occurred (data not shown).

To validate the knockdown of the CRMP2 expression in the Jurkat cells, western blotting was used. First proteins were isolated at two different time points after transfection (48 and 72 hours) using the NP40 buffer. Next, the protein concentrations were determined and western blotting was performed on the protein lysates (10 μ g) from the Jurkat cells (Figure 11A). β -actin was used as a loading control. The expected molecular weight of CRMP2 is between 55 and 65 kDa, and that of β -actin lies around 43 kDa (datasheet antibodies). The total lab quant analyses tool was used to correct for possible differences in loading. To determine the percentage of CRMP2 knockdown, the expression of CRMP2 was compared between Jurkat cells transfected with CRMP2-siRNA and Jurkat cells transfected with scramble-siRNA. The percentage of CRMP2 expression of the Jurkat cells transfected with scramble-siRNA was set at 100%. When the Jurkat cells were transfected with 67 nM siRNA, no differences could be observed between the two conditions (data not shown). At a concentration of 100 nM, there was a only minor difference between the conditions after 48 hours of transfection (Figure 11B, left side), whereas 72 hours after transfection a major decrease could be detected in the expression of CRMP2 in Jurkat cells transfected with the CRMP2-siRNA compared to Jurkat cells transfected with the scramble-siRNA (23,6% versus 100%) (Figure 11B, right side).

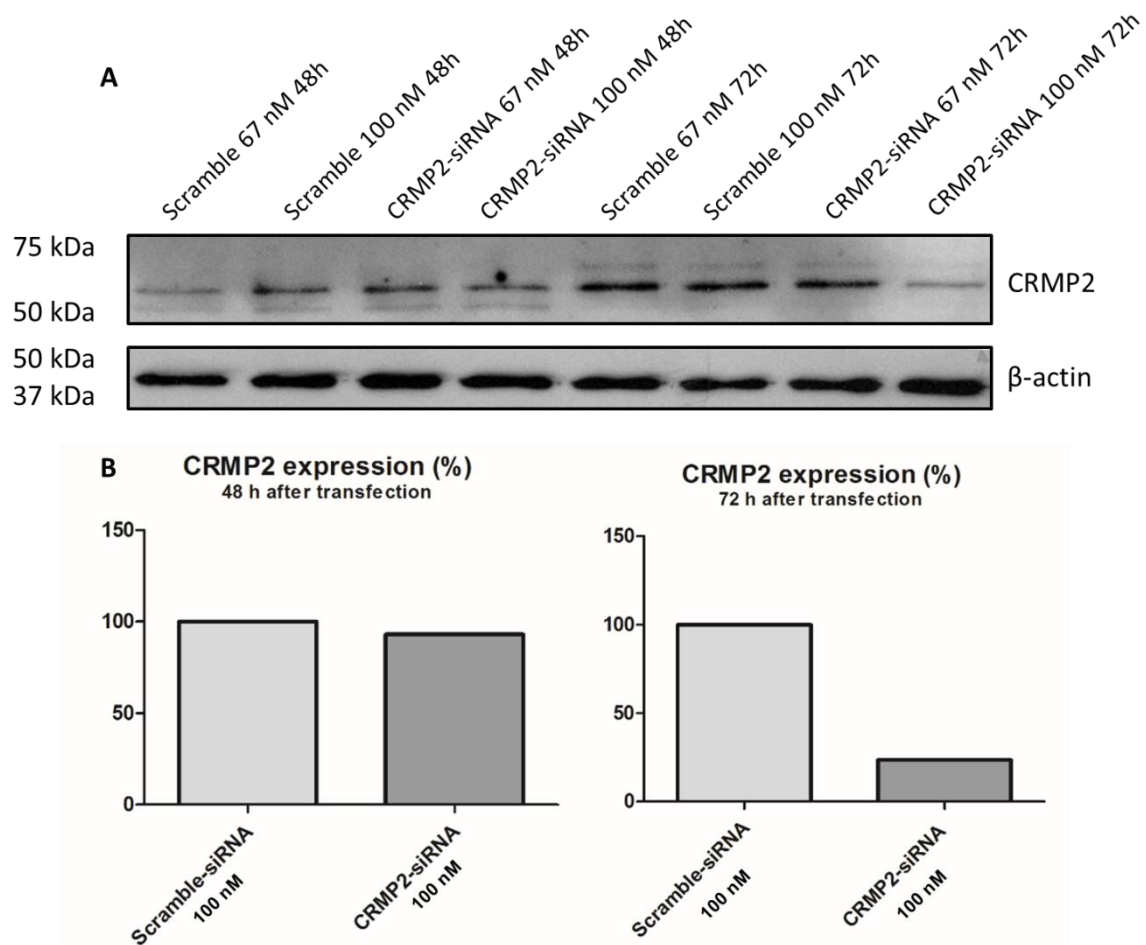


Figure 11: siRNA-induced knockdown of CRMP2 in Jurkat cells. (A) Western blot analysis of CRMP2 expression in protein lysates (10 µg) collected from Jurkat cells 48 and 72 hours after transfection. Jurkat cells were transfected with either 67 nM or 100 nM of scramble- or CRMP2-siRNA. B-actin was used as a loading control. (B) Comparison of the percentage of CRMP2 expression between Jurkat cells transfected with scramble-siRNA and CRMP2-siRNA, 48 hours after transfection (on the left) and 72 hours after transfection (on the right).

Because, only the 100 nM of CRMP2-siRNA resulted in a substantial knockdown after 72 hours of transfection, the experiment was repeated with only this concentration of siRNA. In contrast to the previous experiment, the knockdown of CRMP2 was validated 72, 96, 120 and 144 hours after transfection. This was done to determine the effect of the siRNA on the expression of CRMP2 in the course of time. In this experiment, the same controls were used as in the previous experiment, namely cells transfected with scramble-siRNA (negative control) and cells transfected with the Block-it™ fluorescent oligo (positive control for transfection). To determine whether the cells were efficiently transfected flow cytometric analysis was performed on cells transfected with the fluorescent siRNA. The percentage fluorescein positive cells was approximately 50% and the MFI was approximately 700. These findings indicated that transfection had occurred (data not shown).

To validate the knockdown western blotting was performed on protein lysates from the Jurkat cells (Figure 12A; 120 hours after transfection). B-actin was used as a loading control. After the correction

for differences in loading using the total lab quant analyses tool, the expression of CRMP2 was compared between Jurkat cells transfected with CRMP2-siRNA and with scramble-siRNA. The percentage of CRMP2 expression of the Jurkat cells transfected with the scramble-siRNA was set at 100%. Both 120 and 144 hours after transfection a decrease could be detected in the expression of CRMP2 in the Jurkat T cells transfected with the CRMP2-siRNA compared to Jurkat cells transfected with the scramble-siRNA (79% and 37% respectively versus 100%) (Figure 12B). No decrease in CRMP2 expression could be observed between the two conditions (scramble-siRNA versus CRMP2-siRNA) 72 and 96 hours after transfection.

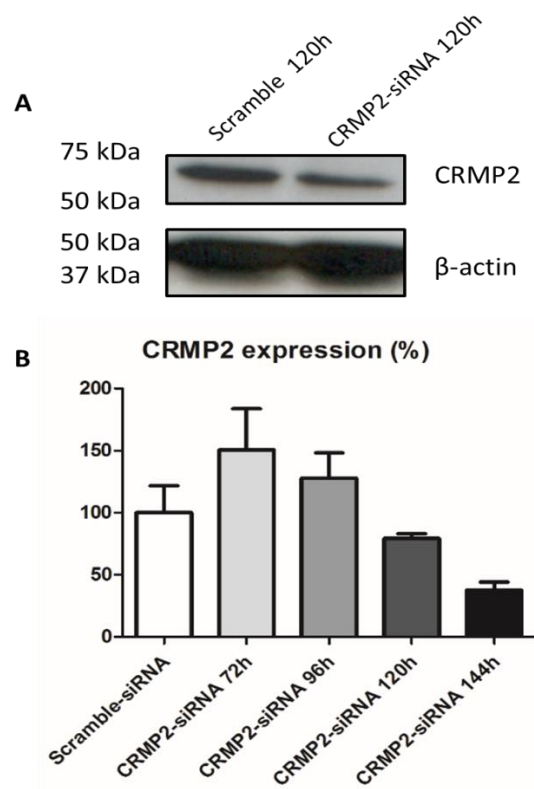


Figure 12: siRNA-induced knockdown of CRMP2 in Jurkat cells. (A) Western blot analysis of CRMP2 expression in protein lysates (10 µg) collected from Jurkat cells 120 hours after transfection. Jurkat cells were transfected with scramble- or CRMP2-siRNA. B-actin was used as a loading control. (B) Comparison of the percentage of CRMP2 expression between Jurkat cells transfected with scramble-siRNA and CRMP2-siRNA at different time points after transfection (72, 96, 120 and 144). The error bars represent standard errors of the mean (SEM).

4 Discussion

MS is a demyelinating autoimmune disorder of the CNS in which T cells play an important role [1, 9]. However, little is known about the underlying disease mechanisms. Recently, a proteomics study was performed in our research group to explore these underlying disease mechanisms of MS. The study revealed 75 proteins, which were differentially expressed in EAE, including CRMP2. Additionally, it was shown by Menon *et al.* that there were elevated levels of phosphorylated CRMP2 present in chronic MS lesions [20]. This phosphorylated CRMP2 was also observed in the brain and spinal cord of patients with active MS lesions and in the animal model of MS [21]. Besides a neural role for CRMP2, this phosphoprotein is also known to play a role in T cell migration. Vuillat *et al.* already illustrated that the expression of CRMP2 in peripheral T cells correlates with the migration of these cells into the CNS during virus-induced neuroinflammation [25]. These findings might indicate a role for CRMP2 in MS. Therefore, we hypothesized that CRMP2 knockdown results in an altered migration rate and differentiation capacity of T cells, which could improve clinical outcome in EAE/MS.

First, the expression of CRMP2 was investigated in T cells infiltrated into EAE lesions. Co-localization of the CRMP2 and CD3 signal was present (Figure 3). Next, the expression of CRMP2 was verified in the human Jurkat T cells and CD4⁺ primary T cells (Figure 4). As expected, both these cell types expressed CRMP2. The expression of CRMP2 was higher in the Jurkat cells, when compared to the CD4⁺ primary T cells. This difference in expression was also observed by Vincent *et al.* [22].

T cells are difficult to transfect [27], therefore it was necessary to optimize the transfection efficiency by means of a fluorescent-siRNA. For this optimization Jurkat T cells were used, although fine-tuning is necessary for all different cell types. Three different aspects of transfection were optimized using the fluorescent-siRNA to obtain the highest transfection efficiency. These three aspects were the transfection method, the effect of different culture media and the effect of activation. The transfection efficiency of the Jurkat cells was evaluated after each optimization step using fluorescence microscopy and flow cytometry.

Three different transfection methods were investigated: electroporation, TransFectinTM lipid reagent and INTERFERinTM siRNA transfection reagent. Vincent *et al.* already transfected Jurkat cells by means of nucleofection [22]. Because, there was no nucleofector available in our university, we opted for electroporation with the ECM 830. Besides nucleofection, also INTERFERinTM siRNA transfection reagent was already described in literature for the transfection of Jurkat cells [26]. We conclude that the TransFectinTM lipid reagent resulted in the highest transfection efficiency (Figure 5-

7). The percentage fluorescein positive cells was approximately 35% and the MFI was around 635. Therefore, TransFectin was used as transfection method in all subsequent experiments.

Secondly, the effect of different culture media on the transfection efficiency was explored. Three different media were investigated: complete medium, RPMI 1640 and Opti-MEM. The RPMI-1640 and Opti-MEM medium were included in the experiment, because it was recommended to use serum-reduced media for the transfections (datasheet TransFectin). Because Jurkat cells were already successfully transfected in Opti-MEM medium [26] and because this medium is recommended for cationic lipid transfections (datasheet Opti-MEM), this medium was included in our experiment. The serum-reduced media resulted in a higher transfection efficiency (Figure 8). Because, the highest transfection efficiency was obtained using Opti-MEM, this medium was used in the experiments that followed.

Thirdly, the effect of activation was investigated since it was already shown in literature that in murine T cells the transfection efficiency was higher after activation [28, 29]. The activation of the Jurkat cells with PMA and Cal caused a major increase in cell death. Approximately 90% of the cells died after using this activation stimulus. This high percentage of cell death can be explained by the fact that activation of T cells can not only cause cell proliferation and differentiation, but also apoptosis [30]. Due to this increased cell death, the results obtained after activation with PMA and Cal were not reliable. Activation with the CD3/CD28 beads for 3, 4 and 5 days resulted in a higher transfection efficiency when compared to Jurkat cells without activation (Figure 10). This was comparable to what was already described in literature for the murine T cells [28, 29]. In summary, the highest transfection efficiency was established when Jurkat cells, activated for 5 days with CD3/CD28 beads, were transfected using the TransFectin™ lipid reagent in serum-reduced conditions.

Due to the fact that each experiment was only performed once, no statistical analysis could be performed. However, we performed similar experiments with just minor adaptations that revealed similar results. Therefore, it was still possible to determine the most optimal conditions.

For the knockdown of CRMP2 in Jurkat cells by means of siRNAs, T cells transfected with scramble-siRNA were used as a negative control. The knockdown was validated by the use of western blotting. A reduction in CRMP2 expression was established using 100 nM of CRMP2-siRNA (Figure 11). After 72 hours there was a knockdown of approximately 76%. In a follow-up experiment the knockdown of CRMP2 was validated 72, 96, 120 and 144 hours after transfection to determine the effect of the siRNA on the expression of CRMP2 in the course of time. For the Jurkat cells there was a knockdown of CRMP2 expression after 120 (21% knockdown) and 144 hours (63% knockdown) (Figure 12B). The

difference between this experiment and the previous experiment (72 hours after transfection), could be explained by the fact that in the previous experiment the CRMP2 expression was much higher when the Jurkat cells were transfected with the scramble-siRNA. When this higher CRMP2 expression was compared to the expression of CRMP2 of the Jurkat cells transfected with CRMP2-siRNA (last experiment), we would also obtain a knockdown of approximately 65%. The results obtained in our last experiment were more reliable as those from the previous experiment, because we included duplicates/triplicates, which was not the case in the previous experiment.

The next step would be a repetition of the previous experiment to confirm the obtained results. Furthermore, CD4⁺ primary human T cells will be included in these experiments. Next, the functional properties of T cells transfected with CRMP2-siRNA will be compared to those of cells transfected with scramble-siRNA. The functional properties that will be investigated are T cell migration and T cell differentiation.

T cell migration will be investigated by means of a transmigration assay, in which the migration of the T cells towards certain chemokines will be explored. The chemokines that will be used are those that play a role in the migration of T cells during EAE and MS, e.g. chemokine (C-C motif) ligand (CCL) 2, CXCL12 and CCL5. We expect that CRMP2 knockdown will cause a decline in T cell migration, because it was already observed that during virus-induced neuroinflammation CRMP2-blockage causes a decrease in the migration of CRMP2-expressing T cells, whereas high CRMP2 levels correlate with a higher migration of T cells [22, 24, 25].

T cell differentiation will be investigated by means of enzyme-linked immunosorbent assays and flow cytometry. The cytokine production will be compared between T cells with and without CRMP2. Before the differentiation capacity can be investigated, the cells need to be activated or differentiated. Only CD4⁺ primary cells will be used in these experiments, because this is the most relevant for our *in vivo* experiments. Furthermore, we do not know the extent to which Jurkat cells can be differentiated. We expect that CRMP2 knockdown will result in a decreased production of pro-inflammatory cytokines (T_H1 and T_H17 cytokines), since the auto-reactive T cells present in MS predominantly have a pro-inflammatory phenotype [10, 31].

To conclude, the highest transfection efficiency was established when activated Jurkat T cells were transfected using the TransFectinTM lipid reagent in serum-reduced conditions (Opti-MEM medium). A knockdown of CRMP2 was established in the Jurkat cells by means of 100 nM of CRMP2-siRNA. After 120 and 144 hours approximately 21% and 63% of the CRMP2 expression was knocked down.

The effect of CRMP2 knockdown on T cell migration and differentiation could not be investigated due to time limitations. Therefore, this will be our first future perspective after we established a knockdown of CRMP2 in the CD4⁺ primary T cells. Next, we will perform an adoptive transfer EAE to investigate whether a knockdown of CRMP2 results in a better clinical outcome (EAE score). This could be expected since we presume that CRMP2 knockdown results in a decreased migration of peripheral activated T cells into the CNS and possibly to a reduced production of pro-inflammatory cytokines, which would be beneficial in both EAE and MS. By unraveling the role of CRMP2 in MS-related T cell functions, this study can lead to new insights into the underlying disease mechanisms of MS and possibly (in the long run) to a new therapeutic strategy for this disease.


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Supplementary data

Protocol 801:



Protocol 801
ElectroSquarePorator™
ECM® 830 HT 25/96 Multi-Well ELECTROPORATION PROTOCOL
Cell Line: Jurkat, subline E6.1
Transfectant: CMV-driven YFP

Cell Preparation:
Cells used for transient transfections were maintained in RPMI 1640 supplemented with 10% fetal calf serum, glutamine (20 mM), and ciprofloxacin (10µg/ml). Immediately prior to transfection, cells were rinsed briefly in serum-free medium and resuspended at 4×10^7 cells/ml in complete ciprofloxacin-containing medium. Aliquots of cells (300µl) were briefly pre-incubated with no more than 30 µg of experimental and vector DNAs, and were transfected in 4mm gap cuvettes or multiwell plates, using a BTX ECM 830 electroporator set to deliver a single 300V, 10ms pulse. After 10 min at room temperature, cells were transferred to prewarmed ciprofloxacin-containing complete medium and allowed to recover for 12 to 16 h prior to analysis.

Electroporation Settings:

Choose Mode:	
Set Voltage:	300V
Electrode Gap:	4mm
Set Pulse Length:	10ms
Set Number of Pulses:	1
Pulse Interval:	na
Electrode Type:	cuvette or multiwell plate
Desired Field Strength:	750V/cm

Electroporation Procedure:

Temperature:	RT
Pulse:	300V, 10ms Press Automatic Start to activate Charge and Pulse Sequence
Post Pulse Treatment:	Transfer to pre-warmed culture medium within 10 minutes.

Results: Within 16 hours of transfection, fluorescent proteins lacking fusion partners are expressed in 80-90% of the viable cells.
Reference: Bunnell SC, et al. 2006, *Molecular and Cellular Biology*

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One Solution.**

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Protocol 918

ElectroSquarePorator™

ECM® 830 HT 25/96 Multi-Well ELECTROPORATION PROTOCOL

Cell Line: human PBMCs (stimulated & resting peripheral blood mononuclear cells), mouse splenocytes

Transcriptant: in vitro-transcribed (IVT) RNA

Cell Preparation: All the cell lines described above were maintained in R/10 (RPMI 1640 (Invitrogen, Inc., Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Biological, Inc., Gaithersburg, MD, USA)). Culture medium (CM) for human T lymphocytes was RPMI with 0.05mMmercaptoethanol, 300 IU/ml IL-2 (Chiron Corp., Emeryville, CA, USA), plus 10% human AB serum (Valley Biomedical, Winchester, VA, USA). Murine splenocytes, collected from 6- to 8-week-old C57BL/6 (The Jackson Laboratory, Bar Harbor, ME, USA) or Pmel-1 TCR transgenic (Tg) mice [14] or Pmel-1 TCR Tg mice crossed with C57BL/6-Sell^{-/-} (Pmel-1 CD62L^{-/-}) [15] were stimulated before electroporation as described below.

For the stimulation of T cells, human PBLs were stimulated with 50 ng/ml OKT3 for 3 days, murine splenocytes were stimulated either with 2 Ag/mlConA for 72h (for wild-typeC57BL/6) or with1mM hgp10025-33 and 30IU/ml IL-2 for 7 days (for Pmel-1 Tg) before electroporation. For the electroporation of unstimulated PBMCs, cryopreserved cells were thawed and washed twice with OPTI-MEM (Invitrogen) and resuspended in OPTI-MEM at 2.5 x 10⁷/ml. The stimulated PBLs subjected to electroporation were also washed twice with OPTI-MEM and resuspended in OPTI-MEM at the final concentration of 2.5 x 10⁷/ml. Cells and cuvettes were prechilled by putting them on ice for N5 min before electroporation. Subsequently, 0.05 to 0.2 ml of the cells was mixed with 2 Ag/1 x 10⁶ T cells of IVT RNA (or as indicated) and electroporated in a 2-mm cuvette (Harvard Apparatus BTX, Holliston, MA, USA), using an ECM830 Electro Square Wave Porator (Harvard Apparatus BTX).

Electroporation Settings:

	Resting Human PBLs	Stimulated Human PBLs	Resting murine splenocytes
Choose Mode:	LV	LV	LV
Set Voltage:	300 V	400 V	300 V
Set Pulse Length:	10 ms	500 μs	5 ms
Set Number of Pulses:	1	1	1
Electrode gap:	2 mm	2 mm	2 mm
Desired Field Strength:	1.5 kV/cm	2.0 kV/cm	1.5 kV/cm

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Electroporation Procedure:

	Resting Human PBLs	Stimulated PBLs	Resting murine splenocytes
Temperature:	4°C	4°C	4°C
Total sample Volume:	100 ul	200 ul	100 ul
Amount of cells:	2.5 x 10 ⁷ /ml	1 x 10 ⁶	2.5 x 10 ⁷ /ml
Amount of transfectant:	5 ug GFP RNA	2 ug IVT RNA	5 ug GFP RNA
Pulse:	Press Start to Activate the Automatic Charge and Pulse Sequence		
Post Treatment:	Immediately after electroporation, the cells were transferred to fresh CM with 300 IU/ml IL-2 and incubated at 37°C.		

Results: The results from optimization experiments altering electroporation parameters for stimulated human PBLs demonstrated that, except at high voltage and long pulse lengths (500 V/2 ms and 500 V/5 ms) the viability of transfected T cells was 63–86% at 24 h postelectroporation (Table 1) and transgene expression of 99% was achieved at 3 days postelectroporation (Fig. 1A). The length of time poststimulation does not significantly influence RNA electroporation as similar efficiencies can be achieved with cells stimulated from 2 to 18 days.

Reference: Zhao, Y. et al., High-Efficiency Transfection of Primary Human and Mouse T Lymphocytes Using RNA Electroporation, Jan 2006, *Molecular Therapy*

TABLE 1: Optimization of electroporation conditions for resting and stimulated T cells

Settings	Resting human PBLs ^a			Stimulated human PBLs ^b			Resting murine splenocytes ^c	
	% GFP	MFI	Viability (%)	% GFP	MFI	Viability (%)	% GFP	Viability (%)
200 V/50 ms	44	170	33	91	746	60	21	9
200 V/20 ms	69	176	62	93	538	78	18	41
200 V/10 ms	69	129	62	83	212	86	17	50
300 V/10 ms	95	286	57	nd	nd	nd	52	28
300 V/5 ms	nd	nd	nd	nd	nd	nd	49	41
360 V/5 ms	88	214	47	nd	nd	nd	56	15
360 V/2 ms	nd	nd	nd	78	437	70	nd	nd
360 V/1 ms	nd	nd	nd	90	153	81	29	52
400 V/0.5 ms	30	24	68	93	100	82	15	57
500 V/2 ms	nd	nd	nd	58	236	54	nd	nd
500 V/1 ms	nd	nd	nd	90	227	63	nd	nd
500 V/0.5 ms	nd	nd	nd	94	123	70	nd	nd
No EP	0.5	8	73	0.6	4	82	0.5	72

GFP expression of CD3+ cells and cell viability (by PI exclusion) were detected 24 h postelectroporation. nd, not determined. Data are representative of two independent experiments.

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Richting: **master in de biomedische wetenschappen-klinische en moleculaire wetenschappen**

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Datum: **10/06/2013**