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DETECTING IL-26 IN MULTIPLE SCLEROSIS PATIENTS BY ELISA

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1. Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is an auto-inflammatory disease of the central nervous system (CNS) that eventually leads to neuro-degeneration. MS affects over one million people worldwide, twice as many women as men, and is the first cause of neurologic disability in young adults(1).

For the patient, the diagnosis of MS means a sentence to hardship, a storm of uncertain hopes and incomprehensible certainty about future loss of function. MS is a progressive disease that can take various clinical courses. In most cases, there is a progression towards accumulation of neurological dysfunction with relapses and remissions (RR-MS). During relapses, symptoms commonly include a loss of sensation, coordination or vision. In the beginning of the disease, there is usually restitution *ad integrum* of neurological functions, but over time, neurological impediments persist even during the remission phases. Thus, the functional losses accumulate as the disease progresses to a second phase (Secondary Progressive [SP-MS]). In ten percent of cases, the disease is progressive from the beginning, without remissions (Primary Progressive MS [PP-MS])(2). In about a quarter of patients, the progression will never lead to major disability, while in some patients the course of the disease quickly evolves towards major functional loss(3), thereby making it a highly unpredictable and heterogeneous disease. The precise cause(s) of MS is/are undetermined, which explains why no curative treatment has so far been developed. There is therefore a huge interest in understanding the immunopathogenesis of MS, as so much is still to be learnt about this autoinflammatory and degenerative disorder of the CNS.

1.1.1 Causes

Multiple sclerosis is thought to occur in genetically predisposed individuals when favorable environmental triggers are present (4).

Epidemiology shows a North-South decrease in MS prevalence in the Northern hemisphere, whereas there is a South-North decrease in the Southern hemisphere, although some disparities exist (1). This variable prevalence is due to genetic differences in different populations but also to other factors (as we will see). Genetic predisposition is now beginning to be characterized with candidate genes mostly coding for HLA II and I molecules (5). More recently, genes coding for other molecules, such as interleukin receptors were identified as genetic risk factors thanks to genome-wide association studies (6)-(7).

The concordance rate for MS in homozygote twins is only 25 % (8) and the fact that moving from a low-risk area to a high-risk area increases the risk of developing MS (9) clearly points towards environmental triggers of the disease. Vitamin D deficiency (10) and smoking (11) have been identified as two non-infectious environmental risk factors of developing MS.

Infections have widely been studied as possible triggers for MS. Becoming infected with measles mumps, rubella, and Epstein-Barr virus at later ages is a risk factor. In this context the hygiene hypothesis that has been put forward in

the case of other immune diseases is referred to (12), although of course it has yet to be confirmed. The most consistent infectious environmental risk factor associated with MS is Epstein-Barr virus (EBV) (13). EBV is a human DNA γ-herpesvirus, which infects B-cells, immortalizes the cells and then remains dormant within. IL 10 has a key role to play in this effect(14). T-cells and NK cells keep infected B-cells from starting monoclonal proliferation induced by the virus. In MS patients, EBV seroprevalence is higher than in control groups (15) and EBV-specific T-cells are increased in MS patients when compared to healthy EBV carriers (16). In particular, our research group has shown that the response of EBV-specific CD8+ T cells was stronger in blood (17) and cerebro-spinal fluid (CSF) (18) of MS patients. Thus, EBV is a possible trigger for MS as well as a possible perpetuating factor of the disease. This may happen through a mechanism of molecular mimicry, where T-cells recognize and react to auto-antigens (for example myelin antigens) that are similar to viral EBV antigens expressed on B-cells by the latent virus(19).

1.1.2 Pathogenesis

The biological course of MS is as variable as its clinical correlate. Autoimmunity causes acute inflammation of the CNS, which is followed not only by repair but also by demyelination and, finally, axonal loss.

The start of MS is thought to correspond to an increased arrival of auto-reactive T-cells into the CNS. This happens across the blood brain barrier (BBB) using integrins (as VLA-4 or α4β1 integrin) and adhesion molecules (20). In the CNS, T-cells secrete cytokines and activate the local innate immune system cells, microglia, astrocytes and monocytes. These then induce local inflammation and are responsible for demyelination and axonal damage. The result is an infiltration of the white matter by lymphocytes, activated microglia and macrophages. There are a multitude of tissue injury patterns and as well as different degrees of inflammation seen in the CNS of MS patients, once more reflecting the heterogeneity and complexity of this disease (21).

To reach this point of self-destruction, the auto-reactive cells must first escape the immune system tolerance for self-molecules. This rupture of tolerance is secondary to an immune disbalance, involving both the innate and adaptive immune systems, and in the latter system, both cellular and humoral responses (22).

1.1.3 The role of T-cells and cytokines

The different T-cell subsets play a key role in MS pathogenesis. They have been studied mostly using the animal model experimental autoimmune encephalomyelitis (EAE).

Let us begin with the T-helper cells. Historically CD4+ Th1 T-cells were thought to be the major responsible T-cells. Th1 cells require interleukine (IL) 12 for their differentiation and produce INF, IL-2 and TNF, cytokines that are correlated with disease activity in MS (23) and attract macrophages.

More recent research has led to the discovery of a new subtype of T-helper cells referred to as Th17 cells. Th17 cells require IL-23, TGF, IL-6, and IL-1 for their development and secrete IL-17A, IL-17F and IL-22. IL-17 production is

associated with disease activity in the CNS of MS patients (24)as well as with inflammatory lesions in many other immune diseases (25). Further T-helper cells, such as Th9, which secrete IL-9 for example (26), have also been described as playing a role in MS.

CD4+ Th2 cells on the other hand, produce IL-4 and Il-10. IL-10 inhibits the cytokine production by Th1 cells. In MS patients IL-10 levels have been observed to decrease during the time preceding relapses and to increase when disease activity slows (25).

Current first-line therapies in RR-MS are IFN molecules and Glatiramer Acetate (27) and have clinical benefits, as they both reduce T-cell proliferation and shift the Th1/Th2 balance towards a Th2 response (3).

T-killer cells as well have their role to play: CD8+ Tc cells produce INFγ and TNFα. They can directly mediate cell lysis and do also have a pathogenic role in MS (5)(28).

γδ T-cells are also pathogenic in EAE (29) and are present in zones of active demyelination in MS lesions (30). γδ T-cells are able to recognize antigens directly without MHC presentation. They are cytotoxic towards virally infected cells, tumor cells and CNS glial cells. They are able to lyse oligodendrocytes and secrete INFγ, TNFγ and chemokines (31). Upon stimulation with Il-23 and IL-1, γδ T-cells produce IL-17(3).

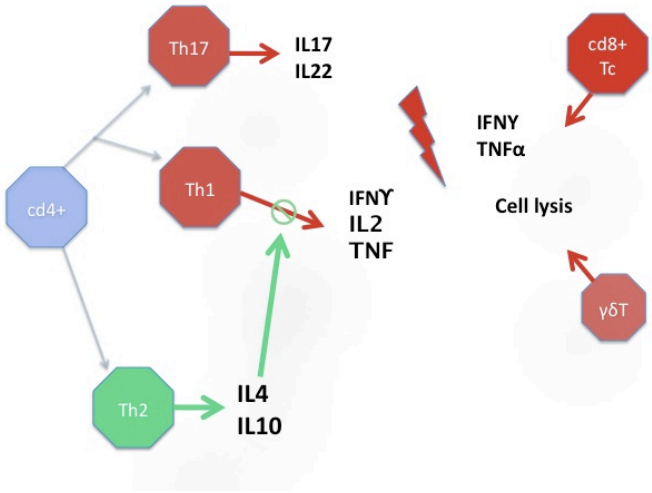


Figure 1. Major immune cells involved in MS disease activity and their cytokine production. Th1, but also Th2 and T17 cells as well as Tc cells produce cytokines that are involved in disease activity and relapses symbolized by the flash arrow.

As we can see, cytokines have a complex but crucial role to play in MS, and some cytokines illustrated here have a role that can be both harmful and protective at the same time (32). Understanding the role of T-cells, their use of adhesion molecules and cytokines to move and communicate will be a key to possible new treatments and to better monitoring of MS in its heterogeneity.

1.2. IL-26

Cytokines are thought to play an important role in the immunopathogenesis of MS (33). Hereafter, we will focus on an as-yet poorly investigated cytokine: IL-26.

1.2.1 What we know about IL-26

IL-26 was discovered in T-cells transformed by herpes virus saimiri (34). Herpes virus saimiri (HSV) is a γ2-herpes virus that transforms T-lymphocytes, immortalizing them in culture(31). Interestingly, in HSV transformed T-cells, one of the only differences with their parental cells is an over-expression of the IL-26 gene (34). Therefore, the HSV-IL-26- T-cells' effect appears to be similar to that of the well-known phenomenon of EBV-IL-10-B-cells (35). Knowing that EBV has been strongly associated with MS, we were interested in examining whether IL-26 may play a role in the immunopathogenesis of MS.

IL-26 belongs to the cellular IL-10 cytokine family of IL-10, IL-19, IL-20, IL-22 and IL-24(36), and is part of the IL-20 subgroup (IL-19, IL-22, IL-20, IL-24 and IL-26)(37) that is thought to protect tissue from pathogen invasion and to trigger the healing of wounds (38). Its gene is located on human chromosome 12q15, close to the genes coding for IL-22 and IFNγ, both important cytokines in MS. Besides, IL-26 was found to be often co-expressed with IL-22(39). The gene of IL-26 has no murine homologue (35).

IL-26 is expressed by various sorts of T-cells at low levels, especially by Th17 cells. It is over-expressed by T-cells after HSV transformation (as mentioned above)(39). It is not expressed by B-cells (34)(36)(40).

IL-26 signals through a unique heterodimeric receptor composed of IL-10R2 and IL-20R1, a combination that is specific to IL-26 (41). As T-cells do not express IL-20R1, they probably are not a target site of IL-26. The complete receptor complex is expressed in different types of non-hematopoietic tissue, such as skin, colon, liver, and heart, all of which are possible targets for autoimmune diseases. Most importantly in the case of MS, the IL-26 receptor complex is expressed in the cerebellum, the medulla and the spinal cord that are common sites of MS lesions (41)(42).

1.2.2 What we still need to know about IL-26

Little is known about the function of IL-26 although there are some hints as to its role in autoimmune diseases.

Indeed, it has been shown that IL-26 increases the secretion of IL-10, IL-8 as well as the surface expression of ICAM-1 (Inter-Cellular Adhesion Molecule 1) on epithelial cells of colon carcinoma and keratinocytes (42). IL-26 expressing T h 17 cells have been found in increased numbers in active Crohn's disease (43). In psoriasis, too, Th17 cells may play a role, even if IL-26 is thought to be more prevalent in the colon or the brain than in the skin (37)(44).

IL-26 follows IL-22's expression pattern in most cases (39)(45)(44). IL-22 has been studied to a much greater degree than IL-26 and is known to participate in the host's defenses against bacteria, fungi and viruses, but has also been involved in intestinal bowel disease [IBD] and psoriasis pathogenesis, liver protection and

other immune diseases (38). Nevertheless, there are some important differences between IL-26 and IL-22: IL-26 does not induce an effect in keratinocytes, nor does it induce the expression of antimicrobial peptides as the rest of the IL-20 subgroup does (37). Furthermore, in contrast to IL-22, IL-26 has an anti-proliferative effect on intestinal epithelial cell lines (38). IL-26 also has the particularity of being active as a monomer or as a dimer (46)(42) and it is inhibited by heparin (42), which might have its importance in *in vitro* assays.

These various research fields reveal what could be an important role of IL-26 in inflammatory diseases and show the current need for still more research to characterize the expression, function and regulation of IL-26 in MS.

1.3 The interest of an ELISA for the detection of IL-26

The laboratory hosting me has recently found that IL-26 secretion by T-lymphocytes from peripheral blood was higher in MS patients than in healthy controls (Figure 2). These findings encourage us to look further in the direction of a role for IL-26 in MS.

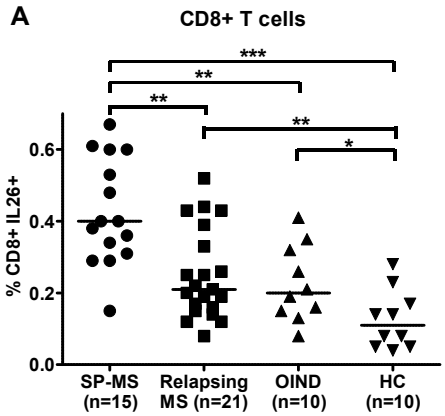


Figure 2. Increased frequency of IL-26 secreting CD8+ T cells during inflammatory and neurodegenerative phases of MS. A: Percentage of IL-26 secreting CD8+ T cells in different categories of study subjets. Each dot represents one study subject. SP-MS stands for Secondary Progressive-MS, OIND for Other Inflammatory Neurological Diseases and HC for Healthy Controls. Horizontal bars represent median values. *P<0.05, ** P<0.01***P<0.001. *Taken from Emilie Jacquiery's thesis, Lausanne 2010, p. 79*

Furthermore, there is no unifying marker of disease activity in MS (47) such as the erythrocyte sedimentation rate used in giant cell arteritis, for instance. Biomarkers in MS would therefore be very helpful, providing information on disease activity, inflammation levels, stages of axonal damage or responses to treatments (48). There is, therefore, a distinct interest in detecting IL-26, both in fundamental research and, later, in clinical activity.

Until now, our group has been looking at IL-26 production by T-cells using flow cytometry. This technique is laborious and can only be applied to a few samples at a time and very few laboratories are equipped with the necessary facilities. We therefore decided to elaborate an enzyme-linked immunosorbent assay (ELISA) capable of detecting IL-26. An ELISA can be used on numerous samples at once, and is less difficult to set up and handle than a flow cytometry assay. This difference will allow a larger scale of MS patients to be tested, taking into

account the different stages and patterns of the disease. The results can then be compared with a sizable amount of controls.

Testing the presence of IL-26 in MS patients with an ELISA is useful for the following reasons:

1) To confirm the previously obtained data about higher IL-26 secretion in MS patients, this time with an easy-to-use method and in a greater number of patients.
2) To find out in which group of MS-patients IL-26 is most often secreted.
3) To test if IL-26 is present in serum/plasma/CSF. It is not known yet whether Il-26 is secreted away from T-cells into the blood or the CSF, or whether it stays bound to the surface of the cell.
4) To discover whether there is a possible use for IL-26 as a marker of certain disease phases. Indeed, preliminary results from our laboratory suggest that IL-26 might be more often secreted during the secondary progressive phase of the disease. Since there is no marker for this stage of the disease thus far, finding one would be of great interest.

The aim of my project is to prepare a functional ELISA assay, capable of detecting IL-26. We will test existing ELISA kits and find out if they are reliable. If there is no such existing ELISA, we will set up our homemade version and test its IL-26 detection in serum and plasma.

2. Material and methods

2.1: Commercial enzyme-linked immunosorbent assays

We started this project by testing two commercial Il-26 detecting enzyme-linked immunosorbent assays (ELISA):

- Human interleukin-26 ELISA Kit, Cat No. HU8305, manufactured by TSZ ELISA in Waltham, MA 02451, USA.
- ELISA Kit for human interleukin 26, Cat No E91695Hu, manufactured by USCN Life Science Inc in Wuhan, China.

Since they had not yet been used in published studies, we first had to make sure they would actually detect IL-26.

2.1.1 Spiking with recombinant and secreted IL-26

We used the two different kits on different days, strictly following the producers' instruction manuals. All the experiences were done in duplicates, and the PhD student and the research assistant permanently supervised me.

To begin we diluted the kits' standards at the different given concentrations to obtain the standard curve. At the same time, we tested the detection of different concentrations of recombinant IL-26 (R&D, cat No. 1375-IL, Lot No. GVG01), diluted in the kit's diluent for the USCN kit, and in R10 for the TSZ kit, within the kits' given range of detection. The PhD student did the experience again. We also e-mailed one producer (USCN) and called the second one (TSZ), to see if they had

an element to correct our way of using their kits. They were unable to indicate any form of misuse.

We then used the two kits on the supernatant of 5*106 peripheral blood mononuclear cells (PBMC) from a healthy donor, kept frozen at -70°C and slowly thawed at 37°C. Living cells were sorted out using the violet LIVE/DEAD stain kit (Invitrogen), and tested with and without stimulation by phorbol myristate acetate and ionomycin (PMA/IONO) for a period of 1, 3, 6 or 12 hours. PMA/IONO stimulation was used to increase CD4+ and CD8+ IL-26 production (as shown by E. Jaquiéry in her thesis and in (49)-(50)).

In the last step we designed a competitive inhibition ELISA. A competition ELISA is an assay in which an antibody binds to the detected antigen. The antigen therefore no longer binds to the kit's detection antibody, thereby decreasing its measured concentrations. We tested the detection of IL-26 in supernatant of PBMC from the same healthy donor, stimulated 18 hours in PMA/IONO. To do the competitive inhibition, we added different concentrations (volumes of 0, 2, 5, 5, 10, 20, 50 and 100l antibody in 250l of supernatant) of a polyclonal anti-IL-26 antibody (Millipore, cat No. 06-1081, Lot No. NRG1719646).

2.1.2 Serum

To test the two kits on serum, we used samples from four MS patients and two healthy controls for the TSZ kit and one MS patient and one healthy control for the USCN kit. We chose patients whose sera were kept in our biobank, frozen at -20° C, and whose T-cells we knew to produce IL-26 thanks to previous tests using flow cytometry. We allowed the serum to thaw at room temperature and used them as soon as they became liquid. We strictly followed the instruction manual to test the sera, with and without dilution in phosphate-buffered saline solution (PBS).

2.2: Homemade enzyme-linked immunosorbent assay

A short look back at this stage reasonably told us that there was no reliable way of using one or the other commercial ELISA kit (see the *Results* section). We decided to use our own homemade IL-26 detecting ELISA.

2.2.1 The ELISA

2.2.1.1 Principle

Our homemade assay is an indirect sandwich ELISA, created by using a coating antibody (Ab) and two detection antibodies. This ELISA allows the quantitative measurement of IL-26 in a human sample, serum, plasma, CSF, etc.

To begin with, we coat the wells with polyclonal anti human IL-26 antibodies (see section 2.2.1.1) (Abs). We then saturate non-specific binding sites with bovine serum albumin (BSA) before adding the sample containing IL-26. Next we add a monoclonal anti human IL-26 antibody that binds itself to IL-26 in the sample. This antibody is detected by another antibody, which is biotinylated. Biotin binds to extravidin. We then put extravidin conjugated to alcaline phosphatase (AP) into the wells. Between

each step the wells are rinsed out. There is then AP in the wells in which antibodies managed to stay, bound to IL-26. At last the AP's substrate is added and reveals a coloured product.

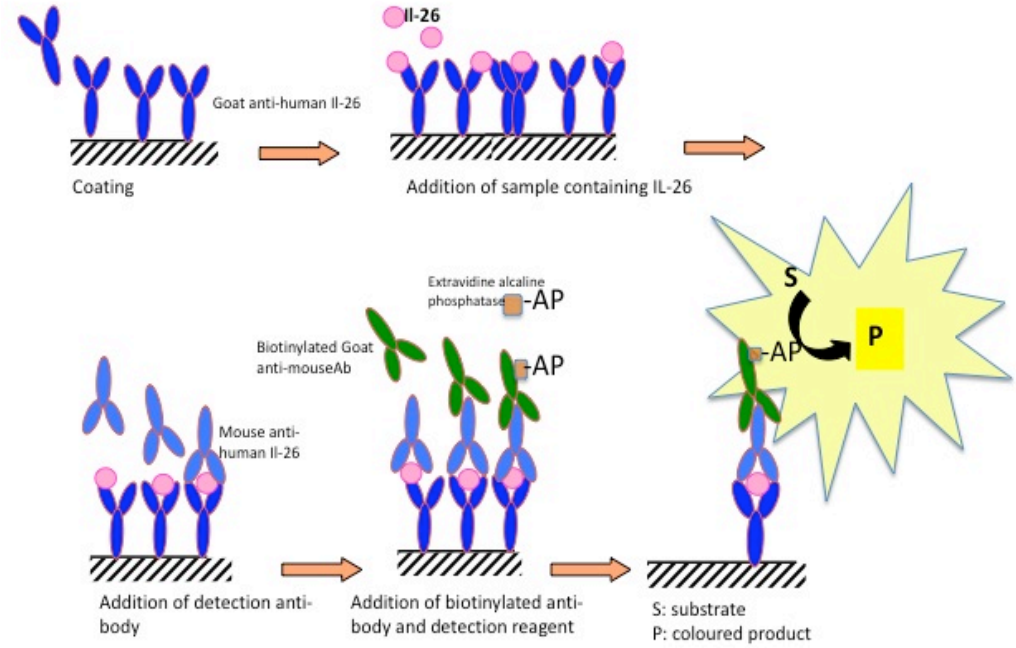


Figure 3. Principle of an indirect sandwich ELISA. Dark blue Y shaped figures stand for the polyclonal goat anti-human IL-26 used for coating. Before adding the samples, unspecific binding sites are saturated with bovine serum albumin (BSA), not shown. Then the sample containing IL-26 is added. And IL-26 binds to the coating anti-body. Pink disks symbolize IL-26. Light blue Y-shaped figure stand for the monoclonal mouse anti-IL-26, the first detection anti-body. A second biotinylated detection anti-body, here the green Y-shaped figures, binds to the first detection anti-body. Extravidine, the orange squares, tied to alkaline phosphatase (AP) is added and binds to the biotin of the second detection anti-body. Then alkaline phosphatase transforms the substrate (S) added in the last step into a colored product (P) measured by a spectrophotometer.

This ELISA is a specific and quantitative test. The concentration of IL-26 in the sample is proportional to the emitted color. The absorbance has to be read when the emission reaches a certain ceiling and the standard concentration curve has achieved a satisfactory degree of steepness.

2.2.1.2 Material and Solutions

- Plates**:** 96 well Nunc Maxisorp Immunoplates, Cat. # 439454
- Coating solution: 15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, pH 9.6
- PBS-T: 137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.05% Tween 20, pH 7.4
- Blocking solution: 1% BSA Fluka Cat. # 05477, PBS-T
- Standard: recombinant IL-26 diluted in PBS-T-1% BSA, 200ng/ml, 100ng/ml, 50ng/ml, 25ng/ml, 12,5ng/ml, 6,25ng/ml, 3,125ng/ml 1,56ng/ml
- Coating antibody: Polyclonal goat anti-human IL-26 Ab, R&D catalog n° AF1375, lot n°IEQ01.
- Detection antibodies:

1. Mouse anti-IL 26 monoclonal Ab, R&D, Catalog n° IC002P Lot n° LGZ23, clone 11711.
2. Goat anti-mouse biotinylated Ab (R&D)

- Detection reagent: Extravidine-Alkaline Phosphatase (AP), Sigma Cat. # E-2636.
- Substrate Buffer: 1M Diethanolamine, Merck Cat. # 116205, 1mM $MgCl_2$, pH 9,8, stored at 4°C protected from light
- Substrate solution: p-nitrophenyl phosphate, Sigma Cat. # 104-105, 1mg/ml in substrate buffer

2.2.1.3 Set-up

The protocol was first established to detect IL-26 in the supernatant of PBMC, on the basis of a protocol for the detection of IL-24 using other antibodies. We used a polyclonal antibody to coat the wells to ensure that all the IL-26 would be captured while a monoclonal antibody, which allows precise quantitative measurements, was employed for its detection. We extended the blocking time and multiplied the numbers of washes after the addition of AP-extravidin, in order to increase specificity. We used recombinant Il-26 (R&D) for the standard curve and as positive controls; R10 was used for the dilutions and as a negative control.

We tested the same protocol on undiluted serum, at two different time points.

In the last experiments, we used the ELISA on plasma, using the same protocol.

2.2.1.4 Final protocol

Day 1

1. Coat each well with 50µl of goat anti human IL-26 diluted 1:25 in coating solution. →Incubate 2 hours at 37°C.

2. Following adsorption, remove the liquid off the well. (Don't wash.)

3. Block non-specific binding sites by filling the wells with 200µl PBS-T-1%BSA. →Incubate for 2 h at 37°C.

4. Wash 3 times with PBS-T.

5. Add standard and samples, 100µl serum/plasma per well, plus 50µl PBS-T 1%BSA per well in the samples → Incubate overnight at 4°C.

Day 2

6. Wash 3 times with PBS-T.

7. Add to each well 50µl of the first detection antibody (mouse anti IL-26) diluted 1:250 in PBS-T-1%BSA →Incubate for 1 hour at 37°C.

8. Wash 3 times with PBS-T.

9. Add 50µl of the second detection anti-body (goat anti mouse biotinylated antibody) diluted 1:3000 in PBS-T-1%BSA → Incubate 1 hour at 37°C.

10. Wash 3 times with PBS-T.

11. Add the detection reagent at a dilution 1:10'000 in PBS-T-1% BSA →Incubate for 30 minutes at 37°C.

10. Wash 6 times with PBS-T (important).

12. Add 50µl substrate solution per well. Develop at room temperature, away from light, without seal

13. Read absorbance at 405 nm, simple filter, at 15min, 30min, 45min, 60min, 75min, 90min (compare the slopes of the standard curves at these different time points).

2.2.2 Samples

Samples from patients were taken from the group's existing biobank after receiving written consent from patients in accordance with our institution's review board guidelines. PBMC were kept frozen at -70°C, and dead cells were excluded when thawed using the violet LIVE/DEAD stain kit (Invitrogen). Serum and plasma were kept frozen at -20°C. PBMC, serum and plasma from healthy controls were frozen before use.

The detection in serum was done twice, 3 months apart. The detection in plasma was done at two different time points and by two different people.

2.2.3 Study subjects

2.2.3.1 PBMC supernatant

We used cells from two relapsing-remitting multiple sclerosis (MS) patients, two secondary-progressive MS patients and two healthy controls. Cells with and without heparin were incubated for three hours. Half of the cells' supernatant was then tested after 18 hours of stimulation with PMA/IONO while the other half was tested without.

2.2.3.2 Serum

We recruited 24 relapsing-remitting MS patients, four patients who had suffered from a clinically isolated syndrome (CIS) suggestive of MS, 11 secondary progressive MS patients and three primary progressive MS patients. The control groups consisted of 13 healthy controls and four patients suffering from other neurological diseases (OND), here progressive multifocal leukoencephalopathy, neurosyphilis, neuro-lupus and Alzheimer's disease.

Table 1. Clinical data of the 42 patients and 17 negative controls involved

Category	Age at blood draw in years [a]	Delay between disease onset and blood draw in years
RR-MS (24)	38±9.5	7±10.25
CIS (4)	31.5±5.75	1±0.38
SP-MS (11)	52±10.25	22±14.25
PP-MS(3)	47±6	4±2
OND (4)	61.5±11.75	0.1±1.5
HC (13)	25±4	n/a

[a]Median±inter-quartile in years

2.3.3 Plasma: first transversal study

We included the same patients already enrolled for the serum assay test and used the plasma from the same blood sample used for the serum. For the control group, we included the same OND patients and used the same blood sample used for the serum experiment. We included new healthy controls, because we were not able to take two blood tubes from each healthy control. The median age from the new healthy control group was 25±6 years this time.

2.2.3.4 Plasma: repetition of the transversal study

First another performer did the ELISA again, using the same protocol, on plasma of 70 new study subjects. We enrolled 10 RR-MS patients, 10 CIS patients, 10 SP-MS patients, 10 PP-MS patients, 10 MS patients during a relapse, 10 OND patients and 10 healthy controls. Then the protocol was applied for the third time, on plasma from 20 study subjects. This time we enrolled the five patients with positive results in the first serum experiment, along with 3 RR-MS patients, 3 CIS patients, 3 SP-MS patients, 3 OND patients and 3 healthy controls.

2.2.3.5 Plasma: longitudinal study

We included 3 patients in the longitudinal study. Two patients with positive results in the first plasma assay were chosen as well as one new patient whose multiple plasma samples were available to us in the biobank. Samples from the three patients were taken at different points in time over the past few years. Regarding the first positive patient, 14 samples were taken between June 2007 and June 2011. With the second positive patient, four samples were collected between August 2008 and July 2010. As for the new patient, 12 samples were taken between December 2004 and January 2011.

2.2.4 Statistical analysis

Differences between two groups were tested using Mann-Whitney test, paired samples were tested with the Wilcoxon test. A p value $p<0.05$ was considered as significant. Correlations were analyzed with the Spearman's test for non-parametric populations.

3. Results

3.1. The validity of commercially available ELISA

During spiking, the detected concentration did not rise with the increase of recombinant IL-26 concentrations either in the TSZ or in the USCN kit. In the USCN kit, the recombinant IL-26 gave a lower absorbance than the zero (Figure 4).

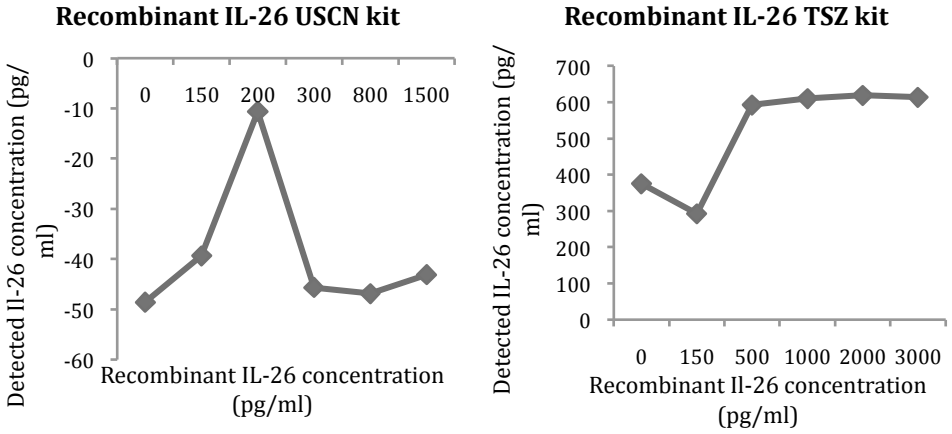


Figure 4. Spiking with recombinant IL-26. Il-26 concentrations were calculated using the two kits' respective standard curves obtained with their standard products. ***Figure 1a****. USCN kit spiking:* recombinant IL-26 was detected in negative concentrations ***Figure 1b****. TSZ kit spiking:* calculated concentrations do not show an increase proportional to the recombinant IL-26 concentrations.

These results were worrisome, strongly suggesting that these commercial kits were utter failures. Nonetheless, we still wanted to find out whether the commercial kits detected natural IL-26 secreted by cells, even if recombinant IL-26 was not detected. Therefore we stimulated PBMC from known IL-26 producers with PMA/IONO. In the end the two kits detected an unknown substance and the absorbance was higher in the wells with stimulated cells as compared to the ones containing un-stimulated cells, especially in the USCN kit (Figure 5). Nevertheless, results in the range of the micrograms are surprising, very unlikely to result from the detection of a cytokine in blood, usually found in picograms/ml.

Now our question was the following: were the kits only detecting naturally secreted IL-26 and not recombinant IL-26, or were they detecting something else? To answer our question, we designed a competition ELISA as described in *Material and Methods*. If the detected substance was IL-26, the detected concentrations had to decrease with the increasing anti-IL-26 concentrations. This was not the case (Figure 6) in either of the two kits.

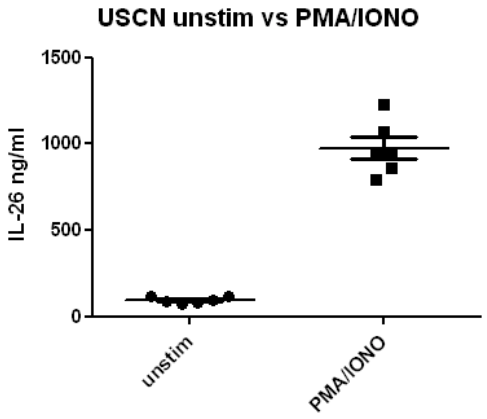


Figure 5. Supernatant from PMA/IONO stimulated cells secrete something detected by the kits. The horizontal bars represent the mean concentration and the standard deviation interval. Detected concentrations are higher in the stimulated cells' supernatant than in that of the un-stimulated cells.

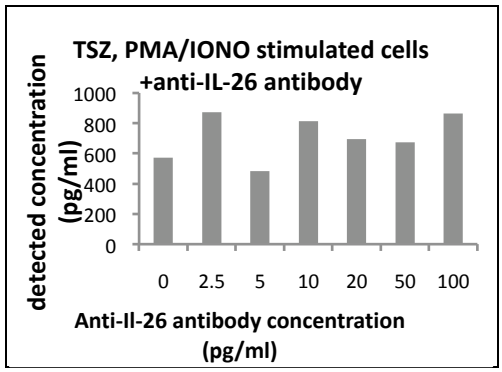


Figure 6. Concentration of detected cell-produced Il-26 with rising anti-IL-26 concentrations. The concentration of the detected substance does not decrease with the increase of polyclonal anti-IL-26 antibody.

Results obtained with the USCN kit were comparable.

As a last resort, we decided to test the two kits on serum, since both of them were initially designed to detect IL-26 in serum. We wanted to see whether there was a difference between the sera of healthy controls and the sera of MS patients. The latter were selected based on the amount of IL-26 detected on the surface of their T-cells using flow cytometry (see Figure 2). We also tested diluted and undiluted sera.

Neither kit detected a rise of concentration in the presumed IL-26 patients. The TSZ kit detected the same concentration whether the sera were diluted or not.

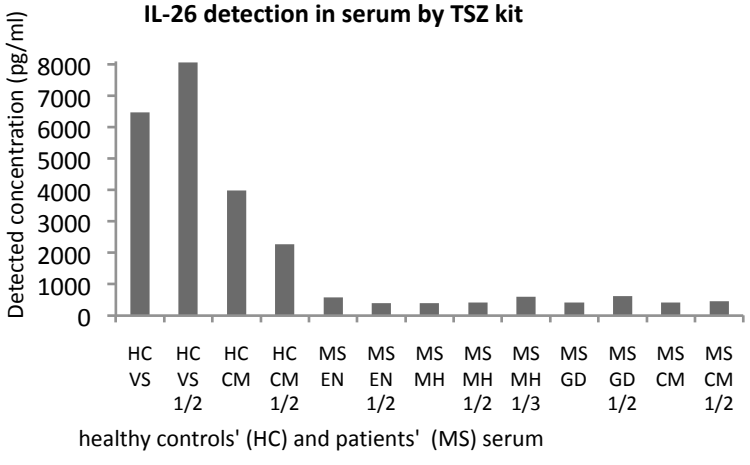


Figure 7. IL-26 detection in serum by the TSZ kit. ½ and 1/3 stay for the diluted sera. Abbreviations stand for the patients' code names. HC stands for Healthy Controls, MS for Multiple Sclerosis patient. The detected concentration does not diminish by half or two thirds as expected with the corresponding dilutions. The detected concentration does not rise in the patients known to have cells producing IL-26.

To summarize, the two tested IL-26 detecting commercial ELISAs did not detect recombinant IL-26, neither did they decrease their detection in supernatant of PMA/IONO stimulated PBMC when polyclonal anti-IL-26 was added, nor did they produce values proportional to dilutions in serum. Accordingly, we decided that

we would not use the commercial kits to test a large number of samples as initially planned, since the results would not be reliable.

3.2. Homemade IL-26 detection ELISA

3.2.1 The homemade ELISA detects recombinant IL-26

After setting up the homemade ELISA as described in *Material and Methods*, we read the standard curves at regular time intervals after adding the substrate. Satisfying linear regression curves were obtained at different points in time (Figure 8).

Figure 8A

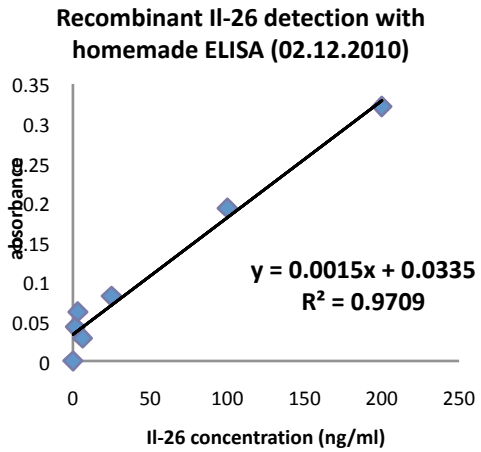


Figure 8B

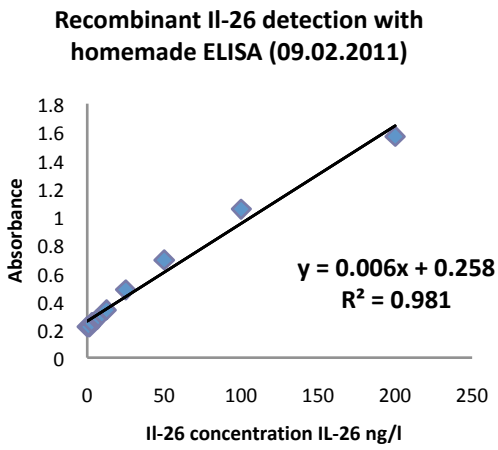


Figure 8. Standard curves obtained in the home made ELISA. Two different assays at different points in time and their trend line equations.

3.2.2 Heparin does not raise IL-26 in PBMC supernatant while stimulation with PMA/IONO does

We tested the secretion of IL-26 by PBMC from healthy controls (HC) and MS patients, with or without heparin incubation and with or without PMA/IONO stimulation.

We found that incubating the cells for 3 hours in heparin did not increase the detection of IL-26 in the supernatant. The tendency was even inversed (Figure 9A). Heparin is known to bind to IL-26 (34)-(35) and was added in order to separate IL-26 from the cell surface. The results suggest that either heparin does not separate IL-26 from cells, or binds to the protein in a way, which makes it undetectable by antibodies.

The supernatant taken from PBMC stimulated with PMA/IONO showed an increase in Il-26 content as compared to the supernatant of unstimulated cell (Figure 9B). The difference was not found to be significant, though.

B

A

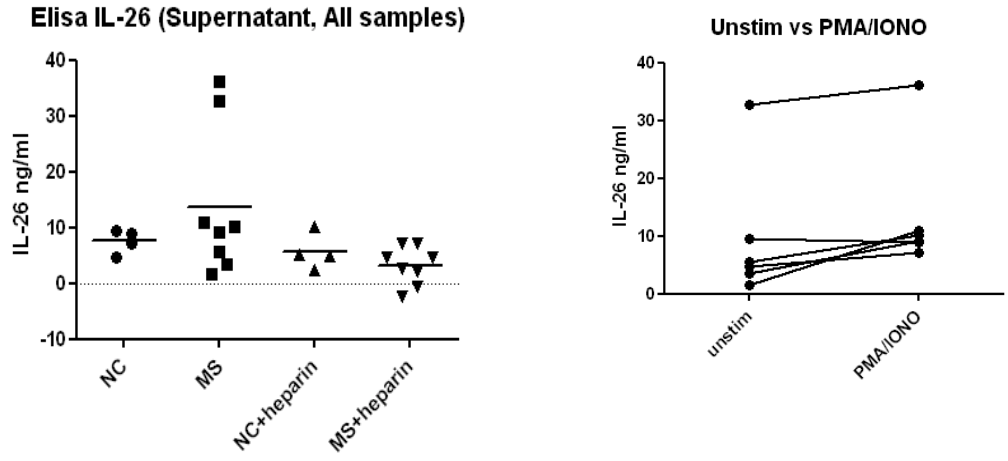


Figure 9. IL-26 in PBMCs' supernatant, with and without 3 hours heparin and with (PMA/IONO) or without (unstim) 18 hours PMA/IONO stimulation. *Figure A* Heparin does not increase IL-26 in the supernatant of cells from Negative Controls (NC) and MS patients (MS); the difference is the other way round. *Figure B*: stimulation with PMA/IONO seems to increase IL 26 in the supernatant (no significant difference was found, P>0.06 with Wilcoxon's test)

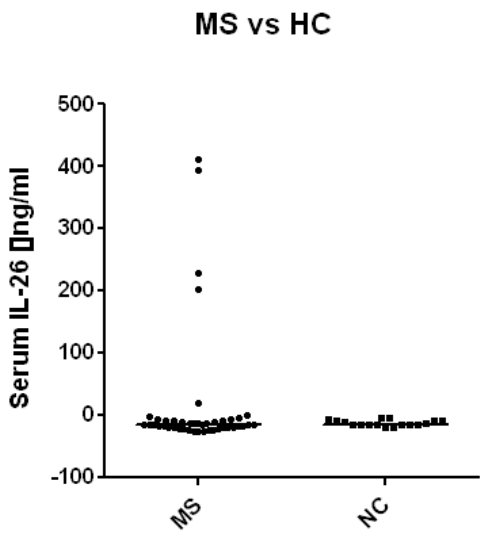
3.2.3 IL-26 is secreted into serum in some MS patients

As we used the ELISA on sera, we detected some IL-26 positive samples (Figure 10):

- In the multiple sclerosis patient cohort, five were positive; four of them were detectable when observed with the naked eye at the ELISA before even reading the absorbance. The patients with positive IL-26 detection were 2 RR-MS, 1 CIS and 2 SP-MS patients.
- No negative controls were found to be positive.

We observed that being positive was not related with duration of disease or with age (Figure 11)

A



B

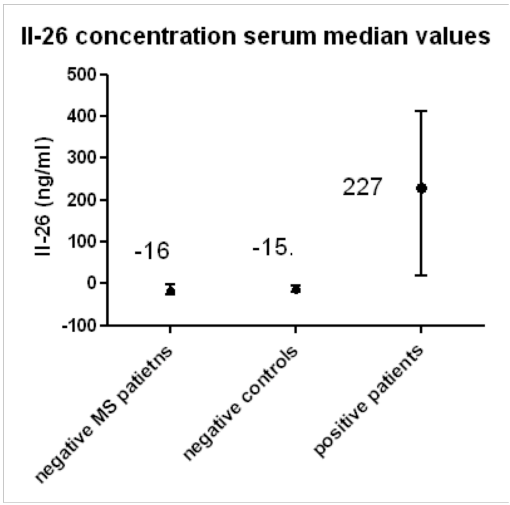
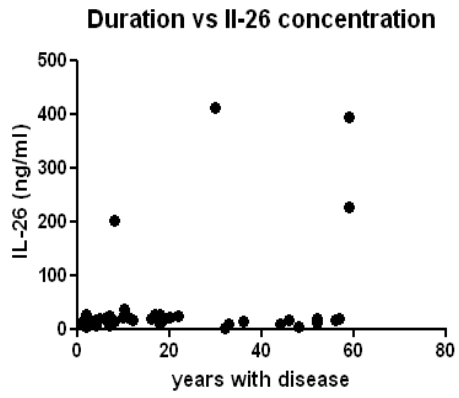


Figure 10. IL-26 concentration in sera from 42 multiple sclerosis patients and 17 controls. *Figure 10A*: "MS" includes RR-MS patients, CIS patients, PP-MS patients and SP-MS patients. "NC" stands for negative controls and includes OND patients and healthy controls. The concentration were obtained with the standard curve y = 0.006x + 0.293,R^2 = 0.949 for the first plate and y = 0.006x + 0.258,R^2 = 0.981 for the second plate, read 90 minutes after adding the substrate. No control was positive. *Figure 10B*: median IL-26 concentrations in negative patients, negative controls and positive patients. Positive patients are highly positive

A



B

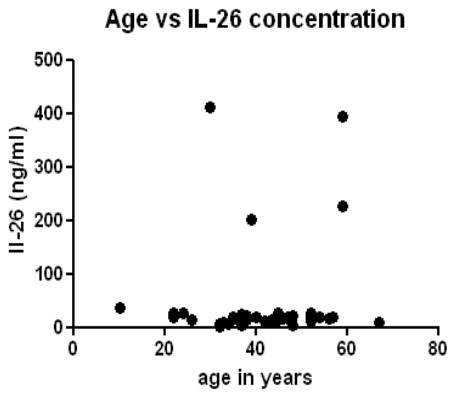


Figure 11. Duration of disease (A) and age (B) at the blood draw vs Il-26 concentration in serum. No correlation was found (Spearmann test).

3.2.4 IL-26 is detectable in plasma

Having found positive IL-26 concentrations in the sera of few MS patients (5/42, 11.9%), we used our ELISA on plasma. Indeed, our biobank is mainly composed of plasma samples, and detecting IL-26 in plasma would be very useful to increase the number of patient samples at our disposal for tests.

We then tested the plasma of the same patients. Four of the five positive patients in the serum assay were positive in plasma. The least positive in serum became negative when IL-26 was detected in plasma (Figure 12).

A B

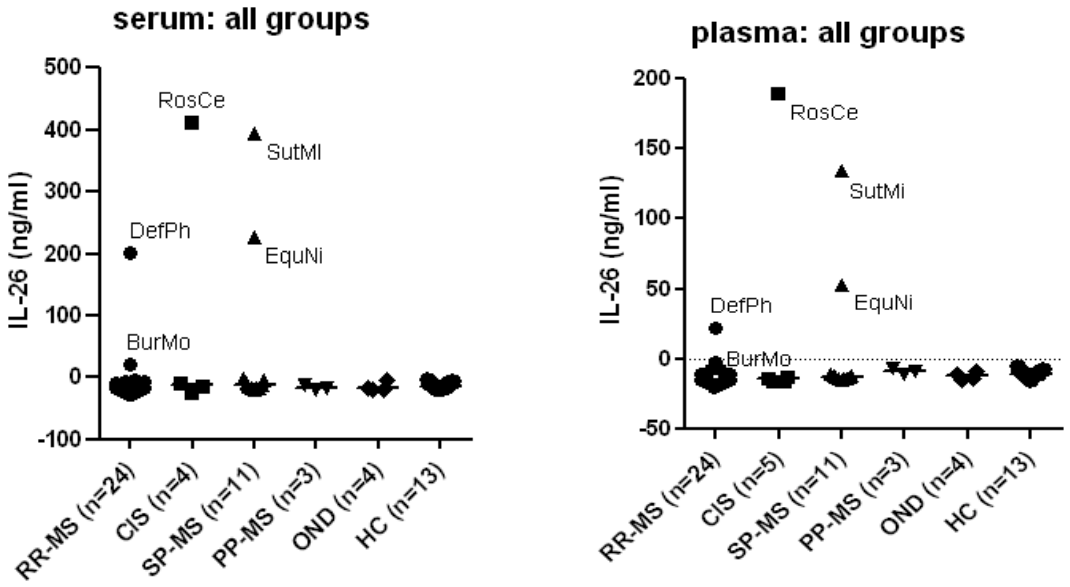


Figure 12. Il-26 positive patients in serum (A) and plasma (B) samples. The positive MS patients are the same patients in the two groups; the least positive serum is negative in the plasma sample. None of the 33 different controls were positive.

3.2.5 Our home-made ELISA proves its reliability

Our ELISA was now ready to be used on more samples. Guillaume Perriard, a PhD student in my host laboratory, tested 70 other samples from patients from different MS categories patients and controls. There was no positive result in any of the samples. He decided to test the same plasma samples tested three months earlier one more. He obtained the same results, with the same four patients showing positive IL-26 concentrations in their plasma on a scale of nanograms/ml.

Detecting the same patients as positive in different samples (serum and plasma), on different time points (same samples tested months apart) and tested by different assay performers, lead us to believe that our ELISA's results are true and reliable, even if IL-26 secretion seems to be a rare event.

3.2.6 Clear patterns in IL-26 secretion are lacking

Our efforts then went to finding out whether there was a recognizable pattern in IL-26 secretion (i.e. whether IL-26 is secreted by all patients at a certain stage of the disease or whether it is found only in a select few but at all times for example). To this end we tested samples from three different patients at different points in time. One patient was positive for the four different samples, the second one was positive in six out of 14 tests (Figure 12), and the last one was never found to have positive Il-26 concentrations in his plasma.

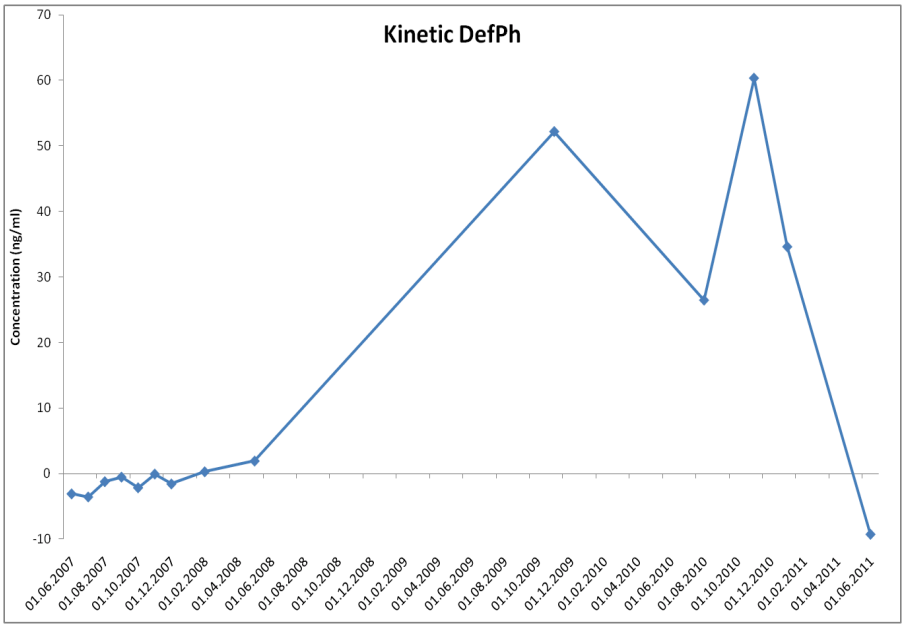


Figure 13. Longitudinal study in an IL-26 secreting MS patient. Numbers are dates of blood draw of the tested samples. Some samples were positive while others had no detectable IL-26 concentrations.

4. Discussion and perspectives

4.1. Using ELISA to detect IL-26 in blood of MS patients is possible

The first point we would like to make is a commercial one: good marketing and a sleek, expensive look are not enough to improve the poor quality of the industrially-produced ELISAs. The disappointing results (see results section 3.1) led us to conclude that if you want something to be done well, do it yourself. On a more serious note, the experiments with the commercial kits left us in a very critical mind space regarding our experiments with IL-26.

As we used our own ELISA in the third experiment on 70 new patients without any positive results (section 3.2.5), we first saw it as a confirmation of well-placed scepticism in IL-26 testing. However, initial disbelief in our protocol was definitively overcome with the experiments that followed: IL-26 was always detected with positive results when re-tested in the positive samples, even with variation of standard curves and background, and this on very different time points, with completely different persons using the protocol. This confirms that the results shown by the homemade ELISA are exact and reliable, in sera as well as in plasma (results 3.2.3-3.2.5). Indeed, serum is the extracellular part of blood after coagulation, while plasma contains fibrinogen and clotting factors. Proteins and particularly small proteins such as cytokines are thought to be more concentrated and easier to detect in serum than in plasma. We were pleased to be able to detect the protein in plasma, since plasma samples are generally more available (as mentioned p.19).

We therefore achieved the declared aim of detecting IL-26 in the blood of MS patients. We can now discuss the use of this detection and whether it is as useful and promising as proposed in the introduction.

4.2 IL-26 detection in blood seems to be specific to MS patients, but rare

Some warnings have to be made before we interpret the results that we obtained with our ELISA. Firstly, criticism about the specificity of the assay could be made. We have no negative control in form of an interleukin close to IL-26 for example. But using a polyclonal and a monoclonal antibody directed against IL-26 makes the ELISA a specific method, very unlikely to detect anything else other than IL-26. The second warning is about the number of samples: positive IL-26 samples were rare (5 altogether on a total of 145 patients and controls). Therefore, any conclusions about the secretion pattern of IL-26 when it is detected as positive have to be considered with caution. Nevertheless, the samples detected as positive where always highly positive with values in the range of nanograms/ml rather then picograms.
However, the experiments brought us some new information:

First of all, IL-26 was secreted in patients from all stages of the disease, with no correlation to the duration of the disease (results 3.2.3). This is opposed to the previous findings about IL-26 being more often secreted in SP-MS patients (Figure 2). With IL-26 being secreted in different stages of the disease and in very few patients, the idea of using it as a marker in MS has to be put aside.

Secondly, with regards to the technique itself, the ELISA does not seem to be the good way of measuring IL-26. Indeed, even if IL-26 is secreted into the blood in nanograms, there is no clear pattern of secretion, and the event appears to be rare (results 3.2.6). It would be of little help to proceed to do large-scale screening of MS patients' blood samples with an ELISA before understanding more about the cytokine and its secretion into blood. IL-26 seems to be secreted in peaks in some patients, while in others it is secreted continuously. This rises many unanswered questions. What could cause IL-26 secretion in some patients to happen at certain points in time and in others continuously? For example, one could imagine that an event stimulates Th 17 cells in a particular way, or otherwise causes a rupture of the BBB. The idea that IL-26 is regularly secreted and then bound to surfaces where it stays undetectable in the blood is another possibility. Indeed, IL-26 is a protein with a large number of positively charged amino acids that can therefore easily bind itself to heparin (39) and possibly even to multiple surfaces such as endothelia or blood cells.

Last but not least, IL-26 detection was specific to MS patients in our results. Indeed, in the 92 MS patients and 53 controls that we tested, only a handful of MS patients (4/92) and no controls whatsoever (0/53) had positive IL-26 concentrations in their plasma. IL-26 secretion therefore appears to be specific to MS patients, at least in the samples we tested. Furthermore, when results were positive, quantities of IL-26 were high. All these findings put together suggest that IL-26 is secreted in relatively copious quantities into the blood, that this secretion is possible in all phases of the disease, and that it takes place either in very few patients or at rare points in time. But it also means that IL-26 is a rare event.

This specific secretion in MS patients and the fact that IL-26 is found in the peripheral blood may support the idea that IL-26 has a promising future, despite the fact that its function in MS is almost completely unknown. In conclusion, the

IL-26 testing is promising, but the use of the ELISA technique is generally inconclusive. Larger testing of patients with this method is not useful at present before we understand more about IL-26.

4.3 Perspectives

The principle aim of further experiments in this domain remains that of confirming the importance of IL-26 in Multiple Sclerosis.

One major problem is that we still lack a firmly established positive control that is due to the following reasons. Firstly, even though we can already detect recombinant IL-26, or produce IL-26 in HSV infected T-cells, we do not yet know how to find a sample in which we would be sure to find IL-26 in natural conditions. Secondly, despite the fact that some patients secrete IL-26, we do not know in which patients to look for this secretion. Furthermore, even if PMA/IONO is known to increase the T-cell IL-26 production(49)-(50), we need to understand how to treat PBMC to provoke their IL-26 production.

Another major challenge is to find a method sufficiently sensitive to be able to measure the occurrence of IL-26 secretion. We know that it is detectable using the ELISA, but this is of little use since IL-26 has here been shown to be rarely secreted into plasma. The use qPCR (quantitative polymerase chain reaction), for example, could prove to be a more precise, alternative method to detect the protein.

Finally, an important way of learning more about IL-26 would be to understand its role. It would be very interesting to confirm which cells host the heterodimeric receptors specific to IL-26, as well as to observe which genes are up-regulated by the cytokine. Understanding its function would therefore extremely useful in imagining how to detect IL-26, in which patients, with which samples and using which method.

Forthcoming challenges can be summarized as follows:

- We must try to detect IL-26 in MS patients through methods other than ELISA or flow cytometry that would allow the detection of RNA rather than the secreted protein, which might be way more sensitive
- We must find a confirmed positive control using the aforementioned method
- We must define on which cells Il-26 acts by confirming on which cells the receptor is located and used
- We must learn more about the expression of genes induced by IL-26 when it binds to its receptor complex, in order to explore its function.

5. Conclusion

To conclude, I would like to evoke the interest I took in following the development of a research project from the start to the end, which is only a new start in itself. At the beginning, we had to overcome the disappointment of discovering the futility of the commercially-produced ELISAs. We then attacked the stimulating task of creating our very own assay. This process was very rich as it allowed me to better understand the specific challenges that are analogous to work in a laboratory and the necessity for a good dose of patience. When finally it came to the interpreting of the results, I fully began to appreciate the importance of a rigorous research structure. It was not always simple to understand how to work in a lab, or to manage the unexpected problems and the valuable surprises that occurred during the course of our experiments. But the excitement of obtaining new, unexpected results and to participate in applied scientific research was highly stimulating. I feel very privileged to have been a part of the project. It goes without saying that I will follow with great interest all future developments in the study of the challenging cytokine that is interleukine-26.

6. List of abbreviations

AB	Antibody
AP	Alkaline Phosphatase
BBB	Blood Brain Barrier
BSA	Bovin Serum Albumin
CIS	Clinically Isolated Syndrom
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein Barr Virus
ELISA	Enzyme-linked Immunosorbent Assay
HLA	Human Leucocyte Antigen
HC	Healthy Control
HSV	Herpes Virus Saimiri
IBD	Intestinal Bowel Disease
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
NC	Negative control
NK	Natural Killer
MS	Multiple Sclerosis
OND	Other Neurological Disease
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline solution
PMA/IONO	Phorbol Myristate Acetate and Ionomycin
PP-MS	Primary Progressive Multiple Sclerosis
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RR-MS	Relapsing Remitting Multiple Sclerosis
SP-MS	Secondary Progressive Multiple Sclerosis
TGF	Transforming Growth Factor
Th	T helper cell
TNF	Tumor Necrosis Factor

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