Neonatal Fc receptor expression in lymphoid and myeloid cells in systemic lupus erythematosus

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Abbreviations: Neonatal Fc receptor (FcRn), systemic lupus erythematosus (SLE), natural killer cells (NK), immune complex (IC), Systemic Lupus Erythematosus Activity Index (SLEDAI), Peripheral blood mononuclear cells (PBMC), phosphate buffered saline (PBS), paraformaldehyde (PFA), mean fluorescence intensity (MFI), interferon- γ (IFNγ).

Abstract

The neonatal Fc receptor (FcRn) is an ubiquitously-expressed protein historically involved in IgG and albumin recycling. Recent data suggest an involvement in the pathophysiology of antibody-mediated autoimmune diseases. Among them, systemic lupus erythematosus (SLE) implies clinical and biological abnormalities of innate and adaptive circulating immune cells potentially involving newly described functions of FcRn.

In this study, FcRn expression was measured by flow cytometry in leukocytes of 41 SLE patients with either active or inactive disease and 32 healthy donors. FcRn expression in B cells, natural killer cells, T cells of SLE patients was statistically lower as compared to healthy donors. Conversely, FcRn levels were statistically higher in non-classical monocyte subpopulation ($CD14^+CD16^+$ monocytes) of SLE patients *vs.* healthy donors. Non-classical monocytes are known to be involved in organ damage in SLE.

Thus, the higher expression of FcRn in these cells could support the hypothesis of FcRn participation in the pathophysiology of SLE, especially in lupus nephritis.

I. Introduction

The neonatal Fc receptor (FcRn) is an intracellular heterodimeric molecule composed of the β2-microglobulin and an α-chain, which is encoded by the *FCGRT* gene. It shows structural similarities with class-I major histocompatibility complex but is not capable of peptide presentation(1). FcRn expression is ubiquitous at the tissue level and has been documented across a wide range of cell types such as endothelial and epithelial cells(2) as well as hematopoietic cells including neutrophils, monocytes/macrophages and dendritic cells(3). The major functions of FcRn encompass the transcytosis and recycling of its two ligands, namely albumin and IgG(4). The recycling of IgGs participates to their extended half-life, which likely depends on their ability to bind FcRn inside macrophages, as demonstrated in mice(5). Aside of these canonical

properties, FcRn plays a role in the enhancement of epitopic repertoire diversity(6) and in humoral immune response(7). It also plays a role in cancer immunosurveillance (8) through its involvement in specific $CD8^+$ T cell antitumoral immune response(9) and *via* its role in natural killer (NK) cell maturation (10). A link between its expression level and tumor size(11) or prognosis (12) has been found in lung cancer patients.

Other data suggest that FcRn is also involved in the pathophysiology of antibody-mediated autoimmune diseases. FcRn-dependent antibody transcytosis and immune complex (IC) cross-presentation appear to be involved in autoimmune mediated glomerulonephritis, as seen in lupus nephritis and Goodpasture (13)(14). In auto-immune nephritis and arthritis murine models that were knocked-out for FcRn, several studies also reported attenuated symptoms, with an absence of specific organ lesions (15,16).

Among autoimmune diseases, systemic lupus erythematosus (SLE) mostly affects women of childbearing age. Its pathophysiology is not yet fully understood but its presentation includes a wide range of clinical and immune abnormalities characterized by a massive production of autoantibodies (17), mainly of IgG isotype. B cells are therefore central players in the pathophysiology of SLE, their withdrawal leading to an absence of organic lesions in murine models (18). Beyond their role in autoantibody production when differentiated into plasma cells, B cells are also involved in the secretion of many pro-inflammatory cytokines and autoreactive T-cell activation (19).

Innate immunity is also impaired with an altered disposal of apoptotic cells by monocytes and macrophages(20). This defect promotes a stagnation of cellular debris favoring the production of autoantibodies. In addition to the qualitative alteration of

their phagocytic function, the expression of CD16 (IgG low affinity receptor FcγRIII) and CD64 (IgG high-affinity receptor FcγRI), in collaboration with FcRn for IC phagocytosis (21), is altered in SLE (22) with a correlation to disease activity and flare(23).

Altogether, the different immune functions of FcRn, particularly (i) the extension of IgG half-life, (ii) the management of IC cross-presentation, and (iii) its ability to increase both the qualitative and quantitative aspects of the humoral response make it an interesting candidate in the pathophysiology of SLE. To follow that hypothesis, we conducted an intracellular measurement of FcRn expression in leukocyte subpopulations from SLE patients with either active or inactive disease, and compared it to a control group in order to determine whether modifications of its expression could occur in SLE, thus explaining some biological features of the disease.

II. Material and methods

The clinical protocol entitled “Role of the FcRn in the pathophysiology of Lupus” (RFPL) is a prospective analysis from Tours University hospital. Patients were enrolled from April 18 to October 31, 2019 after signing an informed consent for the use of clinical and laboratory data. Ethical approval for this study was obtained from the “CPP SUD EST-I” and treatment of the collected data was approved by the local correspondent of informatic and liberty of Tours University. The study protocol is available on clinicaltrials.org under the id: RIPH3RNI18/RFPL.

1. Population

1.1. SLE patients

Patients followed in the internal medicine and nephrology units in Tours University hospital were included in this study by the participating physicians. We enrolled patients aged 18 or older, followed for SLE or with a recently diagnosed disease which meet the international criteria for Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus (24). Pregnant patients, patients under legal protection or suffering from additional autoimmune diseases, except for antiphospholipid syndrome, where excluded from the study. Following inclusion, patients were separated in two groups: (i) newly diagnosed or active disease characterized by a Systemic Lupus Erythematosus Activity Index (SLEDAI) ≥ 4 or introduction of a new treatment, and (ii) inactive disease characterized by a SLEDAI < 4. The clinical and biological data of each patient were obtained after inclusion in the study by consulting their digital medical record. The collected data are summarized in (Table 1).

1.2. Control Group

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers according to institutional research protection guidelines (Agreement N° CA-REC-2015-123) at the Établissement français du sang (EFS) Centre Val de Loire, France.

2. Flow cytometry

One mL of blood sample collected on EDTA was submitted to red blood cell lysis with PharmLyse™ (BD bioscience™). Remaining white blood cells were washed in phosphate buffered saline (PBS), EDTA 2mM. All steps were carried out at 4°C in order to prevent internalization of membrane targets. After an Fc blocking step (FcBlock, Miltenyi™), surface staining was carried out. Two distinct antibody panels were used and identified as leukocyte or monocyte panel respectively. A paraformaldehyde (PFA) fixation step was performed prior to FcRn staining with AF488 conjugated FcRn antibody (R&D™). For this step, Perm Buffer kit (R&D™) was used according to manufacturer instructions.

To analyze FcRn expression in the leukocyte panel, antibodies recognizing surface markers were used as followed: CD45 (APC-Vio770, Miltenyi™) for hematopoietic cells, CD66b (PE, Miltenyi™) for neutrophils, NK cells with CD56 (ECD, Beckman Coulter™), T cells with CD3 (A700, B Biosciences™),$CD8^+$ subpopulation with a CD8 (PE-Vio770, Miltenyi™), B cells with CD19 (APC, Beckman Coulter™) and monocytes with CD14 (Percp-Vio700, Miltenyi™).

In the monocyte panel, cells are stained with CD45 antibody (APC-Vio770, Miltenyi™). CD66b (PE-Vio 770, Miltenyi™) were used to separate neutrophils from monocytes which were identified with an anti-CD14 (Percp-Vio700, Miltenyi™). FcγRIII was stained with an anti-CD16 (APC, Beckman Coulter™). Acquisition was performed with a Gallios™ cytometer (Beckman Coulter™) and the data analyzed with FlowLogic™. In each population, results are expressed as a ratio of FcRn mean fluorescence intensity (MFI) divided by the corresponding isotype MFI.

3. Statisticals analysis

Statistical analysis for data comparisons are performed using GraphPad Prism™ software (version 5.02). Comparisons between groups (newly diagnosed or active lupus disease, inactive lupus disease and control group) are made using a two-tailed t-test without matching due to low sampling. A Welch's correction was used when population variances were statistically different.

III. Results

1. Study population

Forty-one patients were included. The mean age at the diagnosis was 28 years [10-69] and the mean age at inclusion was 40 years [18-77]. Twelve (29.3 %) had an active

disease (SLEDAI 2K ≥ 4) at inclusion, among which 5 (12.2 %) were included at diagnosis. Thirty-six (87.8 %) were female *(Table 1)*. Thirty-two healthy donors were included at the same time.

2. FcRn expression in subpopulations of leukocytes

We conducted the measurement of FcRn intracellular expression in B cells, T cells, NK cells, neutrophils and monocytes in leucocyte populations from SLE and volunteers. As presented in figure 1A, neutrophils, monocytes and NK cells display higher MFI ratio than T and B cells, respectively [3.859], [2.626] and [2.782] vs [2.1] ($CD3^+CD8^+$), [2.011] and ($CD3^+CD8^-$; roughly $CD4^+$) [2.08]. FcRn expression was downregulated in the lupus group *vs* the control group in B cells [2.08] vs [2.749] *(p= 0.0001),* in both $CD3^+CD8^-$ 2.011] vs [2.506*]* *(p= 0.015)* and $CD3^+CD8^+$ [2.1] *vs*. [2.601] *(p= 0.0017)* T cell populations and in NK cells [2.782] vs [3.318] *(p = 0.0090) (Figure 1C)*. No difference was noticed in neutrophils, with a mean ratio of FcRn expression in the lupus group of [3.859] *vs* [4.2] in the control group (p = 0.1671) and in monocytes [2.626] vs [2.354] (p= 0.1564). *(Figure 1C)*. We did not find any difference between active or inactive lupus patients in terms of FcRn expression across cell subpopulations (data not shown).

3. FcRn expression in $CD14^+CD16^+$ non-classical monocyte subpopulation

As MFI ratio value of FcRn expression was increased but not statistically significant in monocytes from lupus patients, we focused on its value in the non-classical monocyte subpopulation. We analysed FcRn expression in these cells, as compared to $CD16^-$ monocytes. In the non-classical monocyte subpopulation, FcRn was upregulated in the lupus group [2.303] *vs* the control group [1.681] *($p < 0.0001$). (Figure 1B).*

IV. Discussion

This is the first study measuring intracellular FcRn expression by flow cytometry in lupus patients in hematopoietic cell populations from whole blood samples, opening the way to study FcRn expression variations in a large panel of diseases.

Whilst evidencing decreased FcRn levels in lymphoid cells (B cells, T cells and NK cells) from lupus patients as compared to healthy controls, overall no variation was observed in myeloid-originating cells (neutrophils and monocytes). However, by focusing on monocytes, which carry out FcRn-dependent specific immune functions, a significant increase in FcRn expression levels in non-classical $CD16^+$ monocyte subpopulation was revealed.

The difference observed between FcRn expression in lymphoid *vs.* myeloid cells could be explained by differences in terms of *FCGRT* gene expression. Indeed it is regulated by factors such as STAT and NFκB (27,28) or Sp1, Sp2, Sp3, c-Fos, c-Jun, YY1,

C/EBPβ and C/EBPΔ(29,30) that interact within distinct regions of the *FCGRT* gene promoter. It is also regulated by a polymorphic variable number of tandem repeats inside intron 1 of the *FCGRT* gene(31), by promoter DNA methylation *via* Zbtb7a and Sp1 factors(32), and by an miRNA (*hsa-miR-3181)* as described by Ferguson et al.(33). All these factors can be involved or not and be differentially expressed and regulated depending on the considered leukocyte population.

Another explanation could be an overall downregulation of FcRn expression in lupic patients' hematopoietic cells associated with a selective upregulation in neutrophils and monocytes (myeloid lineage), the latter using FcRn to carry out their professional immune functions. The higher expression of FcRn observed in the non-classical $CD16^+$ monocyte subpopulation in lupus patients as compared to controls supports this hypothesis. In fact, the non-classical $CD16^+$ monocyte subpopulation itself is altered in SLE and recruited in renal glomeruli through an interaction between IC and the CD16 receptors exposed on cell membranes, bearing proinflammatory functions(34), which could be involved in the development of lupus nephritis(35).

There is also a decrease in monocyte, macrophages(20) and neutrophils(36) phagocytic capacities in SLE(37), leading to a stagnation of cell fragments and IC. This is one of the pathophysiological mechanisms responsible for the generation of autoantibodies. The higher expression levels of FcRn in these cells could participate in the stagnation of IC by recycling them and presenting cryptical epitopes. The stagnation of IC could also directly engage cell surface CD64 on monocytes leading to an increase in local inflammation by stimulating interferon-γ secretion (IFN-γ)(14) and CD16 activation(35), both of these eventually stimulating TNF-α production. IFN-γ and TNF-

α are key players in SLE, especially in the initial stages of the disease and in flare induction (38). Higher FcRn expression levels might, on the contrary, be a reflection of their overproduction, both cytokines being involved in the regulation of FcRn expression(28).

Finally, it is possible that a molecular interaction exists between FcRn and CD16, as it was recently described for CD32a(39). This could participate in explaining FcRn downregulation in cell populations that have lower or absent surface expression of CD16(40). In that case, FcRn could act as an intracellular inflammation sensor. It could be triggered by CD16 and stimulate pro-inflammatory pathways as demonstrated by Gan *et al.* in podocytes with the MAP kinases pathway(41) or by McEwan *et al.* with TRIM21 (another intracellular Fc receptor able to initiate cytokine production in primary immune cells after binding viral particle-containing-IC)(42).

FcRn measurement has recently begun raising interest in human medicine, because of its use in the management of autoimmune disorders(43) as a therapeutic target in diseases involving autoantibodies(44). Strategies blocking the binding of IgG on FcRn, thus decreasing IgG half-life, which is interesting in autoantibody-mediated diseases(45) have given interesting results. The first proof of this concept is the anti-inflammatory potential of intravenous immunoglobulin therapy in a number of autoantibody-mediated diseases(46). The underlying mechanism is a saturation of the FcRn by intravenous immunoglobulins, accelerating pathogenic IgG clearance. Based on this mechanism, new molecules such as efgartigimod are being tested in myasthenia gravis(47) and primary immune cytopenia(44). These promising strategies may bring

need for *ex vivo* FcRn expression monitoring, each disease having its own pathophysiological ways, involving different cell subpopulations. Our measurement method allows for the quantitative measurement of FcRn expression across several cell populations using flow cytometry. It is possible to transpose it into a daily dosing, for the follow-up of patients treated by therapeutic antibodies targeting FcRn, or to study its expression in other antibody-mediated diseases like autoimmune cutaneous diseases(48)(49).

In our study, we did not observe any significant difference in intracellular FcRn expression levels between patients with active, and those with quiescent disease. This may be due to the chosen definition of lupus flare : which was based on a validated criterion, the lupus activity measurement score (SLEDAI), but calculated retrospectively, based on the data found in each patient's digital record and on the physician's decision, either to modify or add a treatment or even to hospitalize the patient, which may have brought some heterogeneity to our sample, in terms of disease severity profiles.

Another explanation for the absence of significant difference may be the various therapeutics used. Indeed, due to the paucity of available data concerning the effects of therapeutics used in SLE on the expression levels of FcRn in the literature, we included the patients, regardless of the ongoing treatments. As these therapies have direct effects on the studied cell populations, it is possible that they also have an effect on FcRn expression levels, either directly or artificially. Moreover, in addition to quantitative alterations of FcRn expression, they may qualitatively affect the binding of FcRn to its ligands by altering the biochemical properties of lysosomes. In fact, all patients included

were treated with hydroxychloroquine, a synthetic antimalarial used in lupus to prevent outbreaks of the disease(25). One of its mechanisms of action consists in a lysosomal alkalization(26), which could impair the pH-dependent functionality of FcRn(4) and modify IgG and IC management by the cells.

In this pilot study, we show an increase in FcRn expression levels in the non-classical $CD16^+$ monocyte subpopulation. Regarding their involvement in organ damage in SLE, we state that this result could support the hypothesis of FcRn participation in the pathophysiological role of this cell contingent, particularly in lupus nephritis. New FcRn-blocking agents could therefore lead to local inflammation and kidney damage attenuation. Either directly, through FcRn-dependent transcytosis blockade and prevention of IC accumulation in the kidney, or indirectly, by decreasing local inflammation and cell activation (by increasing IC clearance). If confirmed on a larger scale, routine follow-up of FcRn could be a promising way of understanding its role in autoantibody-mediated diseases and bring out new perspectives in lupus nephritis care.

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Conflict of interest

None

Patients	**n= 41**
Mean age at diagnosis (year)	28 [10 - 69]
Mean age (year)	40 [18 - 77]
New diagnosis	5 (12,2 %)
Number of women	36 (87,8 %)
SLEDAI < 4	29 (70,7 %)
SLEDAI ≥ 4	12 (29,3 %)
Treatment	
Hydroxychloroquine	36 (87,8 %)
Corticoids	22 (53,7 %)
Methotrexate	1 (2,4 %)
Azathioprine	1 (2,4 %)
Mycophenolate Mofetil	10 (24,4 %)
Cyclophosphamide	1 (2,4 %)
Belimumab	1 (2,4 %)
Rituximab	0
Associated antiphospholipid syndrome	8 (19,5 %)
Clinical manifestations	

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Figure 1. Intracellular expression of FcRn determined by flow cytometry. (A) FcRn expression in neutrophils, B cells, Natural Killer cells, T lymphocytes $CD8^+$ (LT $CD8^+$), T lymphocytes $CD4^+$ (LT $CD4^+$) and monocytes of SLE patients. **(B)** Analysis of FcRn expression in $CD14^+CD16^+$ monocytes in SLE patients and controls. **(C)** Comparison of FcRn expression in SLE patients and control group for each leukocyte subpopulation. Results are expressed as MFI ratio. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *MFI: Mean fluorescence intensity. SLE : Systemic Lupus Erythematosus*